



1967

The Anticholinesterase Activity of Certain Neuromyal Facilitatory Drugs

John Goode

Loyola University Chicago

Recommended Citation

Goode, John, "The Anticholinesterase Activity of Certain Neuromyal Facilitatory Drugs" (1967). *Master's Theses*. Paper 2085.
http://ecommons.luc.edu/luc_theses/2085

This Thesis is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Master's Theses by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.



This work is licensed under a [Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License](https://creativecommons.org/licenses/by-nc-nd/3.0/).
Copyright © 1967 John Goode

**THE ANTICHOLINESTERASE ACTIVITY
OF CERTAIN NEUROMYAL
FACILITATORY DRUGS**



by

John Goode B.S.

A Thesis Submitted to the Faculty of the Graduate

School of Loyola University in Partial

Fulfillment of the Requirements

for the Degree of

Master of Science

February

1967

LIFE

John Goode was born in Paxton, Illinois on April 19, 1939. He attended Paxton High School. He attended both Wabash College in Crawfordsville, Indiana and the University of Illinois at Urbana where he received the Degree of Bachelor of Science.

Upon his graduation from college, the author went to the University of Illinois College of Medicine, Chicago, Illinois, for two years. In the School of Medicine he was a summer fellow in the Department of Pharmacology. In September 1963 he entered upon graduate studies in Pharmacology at the Stritch School of Medicine of Loyola University where he has been a graduate Assistant and a trainee of the National Institute of Health.

ACKNOWLEDGEMENTS

The author wishes to take this opportunity to extend his utmost appreciation to all those who have given him the inspiration and knowledge to achieve this end.

Dr. L. C. Blaber

Dr. A. G. Karczmar

Dr. C. L. Scudder

His Parents

ABSTRACT

Indirect evidence (Blaber, 1963) has suggested that facilitatory drugs may potentiate the muscle twitch and antagonize tubocurarine without inhibiting acetylcholinesterase (AChE). In the present study the indirectly stimulated cat anterior tibialis muscle was used; drugs were injected close arterially (5 micrograms) and intravenously (250 micrograms). At the height of the facilitatory effect, DFP was injected intravenously in doses which completely inhibited AChE in control experiments. The femoral artery and vein were subsequently ligated. One hour later, the muscle was removed and stained for AChE using the Koelle technique. The presence of stain indicated AChE protection by the facilitatory drugs and enzyme inhibition at facilitatory doses. Analogous in vitro studies were performed. Small doses of neostigmine and edrophonium, given close arterially, and larger doses of edrophonium, given intravenously, facilitated neuromuscular transmission without inhibiting AChE. Ambenonium, given close arterially, and ambenonium and neostigmine, given intravenously, inhibited AChE. Methoxyambenonium produced slight inhibition given intravenously or close arterially. Alternate theories to the anticholinesterase theory of the mechanism of action of these compounds are discussed.

TABLE OF CONTENTS

<u>INTRODUCTION</u>	1
Anatomy and Physiology	2
Junctional Morphology	2
Gross	2
Ultrastructure	3
Junctional Transmission	7
Acetylcholine	7
Cholinesterase	9
Pharmacology	13
Neostigmine	13
Edrophonium	15
Oxamides	17
DFP	19
Histochemistry	22
Purpose	24
<u>MATERIALS AND METHODS</u>	25
<u>In Vivo</u> Preparation	26
Preparation for the administration of drugs	26
Recording of mechanical response	27
Experimental procedure	28

Histochemistry.....	29
Preparation of sections	29
Staining procedure.....	30
<u>In Vitro</u> Preparation.....	33
<u>RESULTS</u>	35
<u>In Vivo</u>	39
<u>In Vitro</u>	46
<u>DISCUSSION</u>	52
<u>BIBLIOGRAPHY</u>	63

INTRODUCTION

I. ANATOMY AND PHYSIOLOGY

The mechanism of action of facilitatory drugs at the neuromuscular junction is not yet agreed upon. Disagreement exists as to whether or not the drugs in question inhibit cholinesterase. The drugs, neostigmine, edrophonium, ambenonium and methoxyambenonium are reversible inhibitors of acetylcholinesterase and the degree of inhibition in vivo produced by a facilitatory dose is controversial. The experiments described in this thesis were performed with the objective of obtaining more information on this point. A short account of the anatomy and physiology of the neuromuscular junction precedes a review of the present state of the problem.

A. Junctional Morphology

a. Gross

The motor nerve to the anterior tibialis muscle consists of fibers 1-20 microns in diameter. An impulse travels in this type of fiber at a speed of 5-120 meters per second with a spike duration, including the absolute and relative refractory periods in the order of 0.4-0.5 milliseconds. The negative after potential ranges from 12 to 20 milliseconds and represents 3-5% of the spike height. The positive after potential is 40-60 milliseconds in duration and represents approximately 0.2% of the spike height (Gasser, 1941). The nerve divides approximately seven times and innervates in the order of 120 muscle fibers comprising a motor unit (Sherrington, 1925; Clark, 1931). The units are not compact but are spread throughout the entire muscle. In

most mammalian muscles there is a one to one ratio between nerve branches and muscle fibers; that is, each muscle fiber is innervated by only one nerve branch. The tibialis anterior muscle of the cat has been used for the experiments discussed in this thesis and consists of such focally innervated muscle fibers. At the terminal the axon loses its myelinated sheath (Wagner, 1847). Hubbard and Schmidt (1961) have shown that in the motor nerve terminal the time course of the action potential is 1.3-3 milliseconds, the negative after potential is 15-30 milliseconds, and the positive after potential is 50 milliseconds. These resemble closely the properties of the main axon.

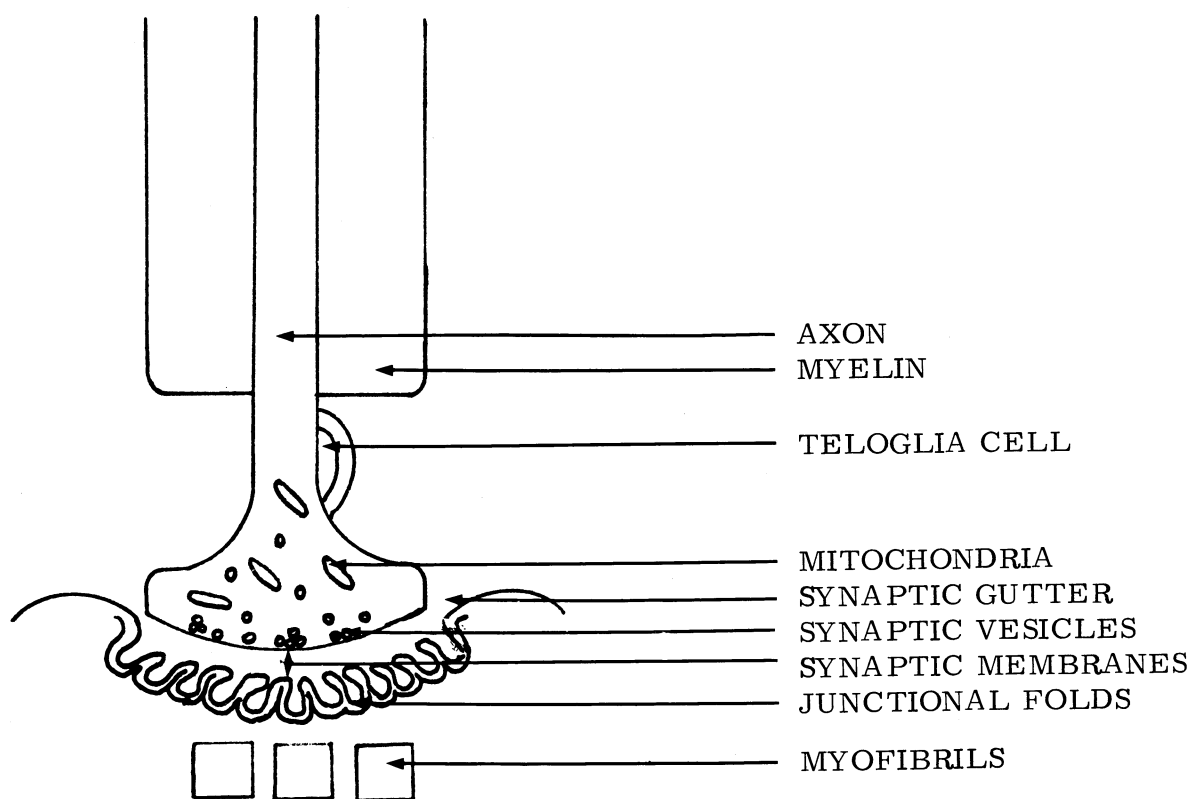
b. Ultrastructure

The study of the fine structure of the neuromuscular junction is of considerable interest for only in the last decade has it been possible, with the use of the electron microscope, to find new elements of structure which begin to provide a morphological basis for the newer understanding of junctional function.

The diagram (fig. 1) illustrates the more important anatomical features of the neuromuscular junction as studied with the aid of the electron microscope. Each of the nerve fibers terminates or lies in a synaptic gutter of the muscle which is made up of a series of foldings called "junctional folds" (Couteaux, 1944). The receptors for the transmitter as well as the enzyme necessary for transmitter destruction are believed to be located in the junctional folds (Koelle and Friedenwald, 1949; Waser, 1959).

FIGURE 1

Schematic drawing of a neuromuscular junction as seen with the aid of the electron microscope.



Synaptic vesicles are found in the prejunctional or axonic endings. These are hollow spherical elements of about 500 \AA in diameter and by chemical methods it can be shown that the transmitter acetylcholine and the choline-acetylase system for the production of acetylcholine are concentrated in the subfraction containing the synaptic vesicles (De Robertis, Pellegrino de Iraldi, Rodriguez de Lores Arnaiz, and Salganicoff, 1962; De Robertis, Rodriguez de Lores Arnaiz, Salganicoff, Pellegrino de Iraldi, and Zieher, 1963). The vesicles are postulated to flow toward the axonic terminal membrane and discharge the chemical mediator into the intermembranal cleft.

The nerve terminals and muscle fibers are separated by continuous membranes (Couteaux, 1960). These membranes show specialization consisting of patches of high electron density called "active points" (Couteaux, 1961). The junctional cleft between the opposing membranes at these points is up to 600 \AA wide (Andersson-Cedergren, 1959). The postsynaptic membrane is depressed at regular intervals into folds that run at right angles to the fiber axis and communicate with the external space at both sides of the nerve terminals. These junctional folds were named the "subneural apparatus" by Couteaux (1947). These folds are certainly a device for increasing considerably the surface of the subsynaptic membrane. This increase has been calculated to be of about 4-5 times (Andersson-Cedergren, 1959). In the frog, the longitudinal spacing of the folds is rather regular and it has been emphasized that the contacting portions of the postsynaptic

6
membrane stain more heavily (Birks, Huxley, and Katz, 1960). They postulate that a special protein layer may be attached to large parts of this membrane. It would be tempting to identify this postsynaptic density as the locus of chemoreceptive function, but without further proof its role remains obscure.

Koelle and Friedenwald (1949) first showed histochemically the existence of a high cholinesterase activity at the level of the motor end-plate. The exact localization was, however, obscured by diffusion artifacts. Couteaux and Taxi (1952) obtained a more accurate localization by slight changes in the technical procedure and found that the structures stained by the acetylthiocholine method are identical with the subneural apparatus previously identified with supravital methods. They concluded that the subsynaptic membrane contributes the major portion of the total cholinesterase activity. However, recent histochemical studies using electron micrographs have shown some cholinesterase to be present presynaptically in the membrane and vesicles (Barnett, 1962).

Interesting studies have been carried out on the neuromuscular junction with the use of radioactive drugs that will bind specifically the receptors normally occupied by the transmitter. Waser (1959), using radioactive curarine, has calculated that one end-plate contains about 10^7 receptors which would occupy only about 2% of the highly folded subsynaptic membrane. These receptors might be localized at certain points of this membrane and not dispersed throughout it.

a. Acetylcholine

It is now generally accepted that acetylcholine is responsible for the transmission of the excitation from the nerve endings to the motor end-plate. Brown, Dale and Feldberg (1936) discovered that acetylcholine was involved in transmission at the neuromuscular junction by identifying it in the perfusion fluid after stimulating the nerve to the hind limb muscles of the cat. Injection of acetylcholine into arteries supplying the gastrocnemius muscle caused a quick contraction of the muscle. It has been confirmed that stimulation of motor nerves in isolated muscle preparations causes the release of acetylcholine (Straughan, 1960). Microelectrode techniques have shown the quantity of acetylcholine necessary to produce a response is 1.5×10^{-15} gm. (Krnjević and Miledi, 1958), which because of the narrow gap at the junction is a concentration of approximately 1×10^{-3} M.

Cholinergic fibers contain an enzyme, choline acetylase, which in the presence of adenosinetriphosphate forms acetylcholine by transferring acetate from acetyl coenzyme A to choline (Nachmanson and Machado, 1943). There is more than enough choline in the plasma to support this synthesis no matter how heavy the traffic of nerve impulses. Choline being a quaternary compound is unable to penetrate nerve axons by passive diffusion so the nerve endings must extract the choline from the extracellular fluid. Birks and MacIntosh (1961) have postulated a choline carrier in the terminal axonal membrane. The release of the transmitter accelerates its formation

but the mechanism of this is not understood. Choline acetylase is found all along the nerve trunk. Acetylcholine and choline acetylase are stored in the synaptic vesicles (De Robertis and Bennett, 1955). Even in the absence of nerve impulses there is a spontaneous release of acetylcholine from the nerve endings giving rise to minute voltage fluctuations in the region of the motor end-plates; miniature end-plate potentials, about 1% of a full end-plate potential (Fatt and Katz, 1952). On the arrival of a nerve impulse there is a simultaneous release of a large number of quanta of acetylcholine. This is caused by a depolarization of the terminal causing an influx of calcium which alters the properties of the vesicles or the axoplasm just inside the membrane so that the vesicles spill their contents into the synaptic gap (Birks and MacIntosh, 1957). Depletion of calcium reduces the output of acetylcholine and the frequency of miniature discharge. Magnesium ions have the opposite effect; an increase in magnesium ion concentration decreases acetylcholine output (Del Castillo and Engbaek, 1954; Hubbard, 1961).

The combination of acetylcholine with its receptors makes the end-plate permeable to all free anions and cations on either side of the membrane so that the local potential in this region tends toward an equilibrium level. This localized end-plate negativity is known as the end-plate potential. When the end-plate potential reaches a threshold level (about $1/2$ resting value in the frog (Nastuk, 1953) or $1/4$ resting value in the cat (Boyd and Martin, 1956)) the membrane is short circuited and surrounding areas of the muscle

3

membrane, still in the resting state, discharge into this current sink and a muscle action potential is initiated and propagates away from the end-plate region (Eccles, Katz, Kuffler, 1941). The amount of acetylcholine released is in excess of what is needed to reach threshold (Brown, 1937), and yet it disappears quickly allowing the end-plate to repolarize before the end of the refractory period of the muscle fiber.

Cholinesterase is responsible in part for the rapid disappearance of acetylcholine but the end-plate potential is still short-lasting in the presence of cholinesterase inhibitors (Eccles, Katz, Kuffler, 1942). Other mechanisms for the rapid removal of acetylcholine, such as rapid diffusion to subthreshold concentration (Ogston, 1955) or resynthesis to a chemical precursor (Abdon and Bjarke, 1945) have been suggested. The presence of cholinesterase may be merely an emergency mechanism for the removal of abnormally excessive amounts of acetylcholine. However, the artificial conditions of some animal experiments may be what cause it to appear unnecessary. (Blaber, 1962).

b. Cholinesterase

The cholinesterases constitute a group of esterases which hydrolyze choline esters at a higher rate than other esters. All esterases which show this specificity are inhibited by 10^{-5} of eserine (Richter and Croft, 1942) and are much more sensitive to quaternary ammonium ions than are other esterases. This outstanding affinity of cholinesterases for cationic

substrates and inhibitors is the most characteristic feature of these enzymes, and suggests that the active center of cholinesterases, in contrast to other esterases, includes a negative group ("anionic site") in addition to the ester-binding group ("esteratic site") (Wilson and Bergmann, 1950).

The existence of an enzyme responsible for the destruction of the humoral transmitter was suspected by Loewi (1921) early in his studies of the "vagusstoff" and later demonstrated by Loewi and Navratil (1926). Later studies showed that those portions of skeletal muscle that had a high concentration of motor end-plates also contained the most acetylcholinesterase (Couteaux and Nachmansohn, 1940). The classic investigation by Marnay and Nachmansohn (1937) demonstrated that there was enough acetylcholinesterase in the end-plate region of the frog to hydrolyze, during the few milliseconds of the refractory period, the amount of acetylcholine calculated to be released from the nerve terminal. Similar studies led to the same conclusions for the toad (Feng and Ting, 1938). Nerve degeneration experiments led to the conclusion that whereas there was some acetylcholinesterase activity in the terminal nerve branches, most of the enzyme activity was localized in the subneural region (Sawyers, Davenport, Alexander, 1950; Brooks and Myers, 1952). Refined microchemical investigations have confirmed earlier conclusions that acetylcholinesterase is intimately associated with motor end-plates (Couteaux and Nachmansohn, 1940).

Considerable work has been devoted to the localization of cholinesterase in muscles. When pure histochemical techniques are applied to normal muscle, cholinesterase is found at the following sites as summed up by Beckett and Bourne (1957).

1. Small classical end-plates arranged in straight rows across muscle fiber or in groups. The cholinesterase in the end-plates is of two kinds, predominantly acetylcholinesterase but also butyrylcholinesterase (Denz, 1953; Holmstedt, 1957). Most of the acetylcholinesterase at the junction is postsynaptic and belongs to the so-called subneural apparatus. Very little is presynaptic (Zacks, 1964).
2. Plates composed of parallel gutters arranged as a "cake frill" or palisade, the gutters being oriented in the same direction as the long axis of the muscle fiber (Couteaux and Taxi, 1952).
3. Large cap-shaped areas situated over the ends of muscle fibers, with gutters arranged in parallel or in a somewhat reticular pattern. These endings are, in fact, situated at the musculo-tendinous junctions (Gerebtzoff, 1959). They are also found where muscle fibers end in the middle of a bundle. The possibility exists that these may not be motor end-plates but stretch receptors--if this is so, then the presence in them of acetylcholinesterase is of some interest.
4. Cholinesterase is also present in muscle spindles, both in the end-plates of the intrafusal fibers and in the fibers themselves (Coers and Woolf, 1959).

Gerebtzoff (1959) has shown that the speed with which a muscle contracts is dependent more upon acetylcholine sensitivity than upon the amount of cholinesterase present.

Koelle and co-workers have indicated that the total neuronal acetylcholinesterase consists of a "functional" portion external to a relatively impermeable cellular membrane and an internal portion. It has been suggested that the internal or "reserve" portion represents the source for the replacement of the functional enzyme of the cell surface and could possibly be identical with the endoplasmic reticulum (Fukuda and Koelle, 1959).

Cholinesterases may be concentrated at some distance from the acetylcholine-releasing structures; these enzymes are not inevitably concerned with hydrolysis of acetylcholine; and a parallelism between acetylcholine amounts and cholinesterase activity is not always present (Gerebtzoff, 1959).

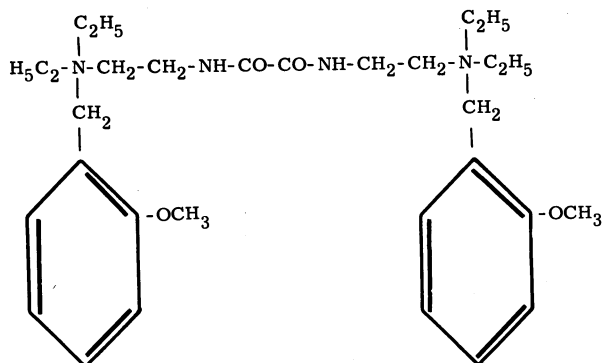
II. PHARMACOLOGY

Numerous compounds capable of facilitating neuromuscular transmission have been synthesized. Many of these have been shown to possess anticholinesterase activity and it was natural to ascribe the effects of these compounds on skeletal muscle to inhibition of the enzyme at the neuromuscular junction with the consequent preservation of the transmitter. In recent years the lack of correlation between the ability of certain of these compounds (See Fig. 2) to facilitate neuromuscular transmission and to inhibit cholinesterase *in vitro* has led to research which shows that some of these compounds cause at least a part of their facilitating action via other mechanisms (Randall, 1950; Wescoe and Riker, 1951; Riker, 1953; Karczmar, 1957; Blaber, 1960; Blaber and Bowman, 1962; Blaber, 1963).

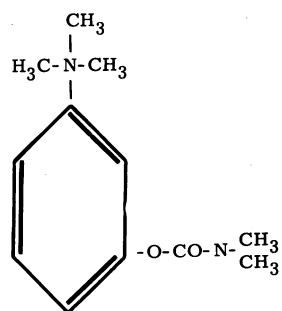
A. Neostigmine

Neostigmine is a synthetic anticholinesterase which was developed as an outcome of the studies of the cholinesterase-inhibiting properties of physostigmine. Although there is little difference between the *in vitro* potencies of neostigmine and physostigmine to inhibit cholinesterase in various species (Nachmansohn, Rotherberg and Feld, 1948), the anticholinergic and twitch potentiating action of the former drug is much more rapid in onset than that of the latter (Riker and Wescoe, 1946). The rapidity of action of neostigmine compared to that of physostigmine can probably be ascribed simply to a more rapid rate of combination with the receptors (Blaber, 1962).

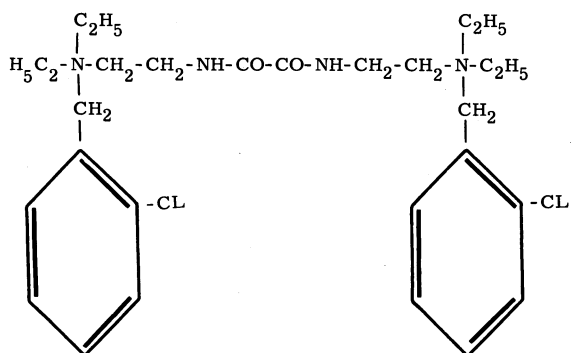
FIGURE 2



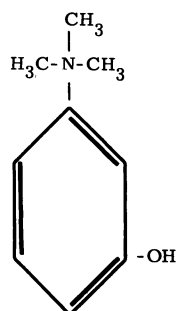
METHOXYAMBENONIUM



NEOSTIGMINE



AMBENONIUM



EDROPHONIUM

In the rat diaphragm, neostigmine antagonizes not only the neuromuscular paralysis by curare but also paralysis by depolarizing blockers such as decamethonium (Secher, 1951). This antagonism of depolarizing blockade does not hold true in the cat though, for an increase of the blockade is observed (Bowman, 1962). Neostigmine is known to have a direct action at the neuromuscular junction in addition to its anticholinesterase action (Riker and Wescoe, 1946), but this has been shown to be too weak to contribute to its action at the neuromuscular junction (Smith, Cohen, Pelikan, and Unna, 1952; Hobbiger, 1952; Blaber, 1962). Berman-Reisberg (1957) showed that neostigmine inhibited choline-acetylase but 8×10^{-3} M was necessary to cause 50% inhibition which is strikingly different from the potency to inhibit acetylcholinesterase, 50% inhibition being produced by 2.8×10^{-7} M, i. e. at a concentration of approximately 40,000 times less. The finding that drugs of this type potentiate the action of applied acetylcholine is merely a demonstration that in sufficient concentration they possess an anticholinesterase action. The question of whether this is the sole, or even the main mechanism of action of these compounds in the usual effective doses is not answered.

B. Edrophonium

The compound edrophonium is a particularly interesting substance because it reveals some of the weaknesses in the experimental methods. It is a quaternary compound which inhibits the enzyme reversibly by combining

with it only at the anionic site, and thus blocks attachment of the substrate (Holmstedt, 1959).

The rapid onset and short duration of action of edrophonium with respect to neostigmine has been noted by a number of investigators (Cowan, 1938; Macfarlane, Pelikan and Unna, 1950; Riker, 1953; Wilson, 1955). The ability of this compound to reverse the neuromuscular blocking action of tubocurarine is well known also, but the earliest experiments indicated that it was only a feeble anticholinesterase, about one-hundredth as potent as neostigmine (Randall, 1950; Hobbiger, 1952). It was of interest to compare the time course of action and potency of this compound in vitro and in vivo, since it had been presumed that the antitubocurarine and acetylcholine potentiating properties of edrophonium and neostigmine are primarily the result of their anticholinesterase activity. Smith, Cohen, Pelikan, and Unna (1952) obtained results which indicated that edrophonium was as much as one-quarter as active as neostigmine. These same workers found that there was no positive correlation between the relative potencies of the compound as an anticholinesterase and its ability as potentiator of acetylcholine on the frog rectus muscle. The inhibition of cholinesterase by edrophonium was reversed by the addition of substrate too rapidly for the time course to be measured with the manometric technique. The evidence obtained neither directly confirmed nor negated the thesis that the potentiation of acetylcholine and antagonism of tubocurarine produced by this compound is the

result of its inhibition of cholinesterase (Smith, Mead, and Unna, 1957).

Wilson showed (1955) that for the acetylcholinesterase of electric eel at 20° C, K_i for edrophonium was 3×10^{-7} as opposed to 10^{-7} for neostigmine. The rate of formation (and of dissociation) for the complex with edrophonium, was much greater than that for neostigmine. Contrary to observations with neostigmine, potentiation of response to acetylcholine is maximal after an exposure to edrophonium for only two minutes or less and decreases subsequently over a period of 10 to 20 minutes until it reaches an equilibrium value. The formation of the enzyme-inhibitor complex in the absence of substrate is completed in 12 seconds. The reverse reaction proceeds rapidly also (one-half dissociation was not more than 30 seconds). The one-half time dissociation for neostigmine is about 12 minutes (Wilson, 1955). If the experiments were performed in equilibrium conditions this should not affect the results, but if the association and dissociation of neostigmine does not have time to reach equilibrium, it may appear very much more powerful than edrophonium; in these circumstances it is, in fact, behaving as a non-competitive "irreversible" inhibitor (Wilson, 1955).

C. Oxamides

Ambenonium chloride (Mytelase; WIN 8077) and methoxyambenonium chloride (WIN 8078) are members of a series of bisquaternary oxamides synthesized in the Sterling-Winthrop laboratories and first reported by

Arnold, Soria and Kirchner (1954). Both are synergistic with neostigmine in mice and both facilitate transmission and oppose blockade in the autonomic nervous system (Karczmar and Howard, 1955). Both compounds have been shown to be reversible inhibitors of acetylcholinesterase (Karczmar and Howard, 1955; Koelle, 1957; Blaber, 1960). In this regard it was found that ambenonium is more than 100 times as active as methoxyambenonium.

Ambenonium dissociation occurs extremely slowly excepting in the presence of relatively high concentrations of certain cations (Koelle, 1957). Ambenonium potentiates the response to the indirect single shock stimulation of cat muscle and antagonizes curare blockade. Although a powerful anti-curare agent, methoxyambenonium does not potentiate the maximal twitch tension (Lands and Karczmar, 1955; Blaber, 1960). Ambenonium will elicit twitch responses on close arterial injection and augment the excitatory and paralyzing action of acetylcholine, succinylcholine and decamethonium on the cat's tibialis muscle. Karczmar (1957) showed that methoxyambenonium produced reversal of the action of succinylcholine and decamethonium on the cat's tibialis muscle. Also, since methoxyambenonium antagonizes paralysis by curare and depolarizing agents as well, Karczmar stressed in his discussion that the current theories on the mode of action of the depolarizers offered an inadequate explanation for the properties of methoxyambenonium. Blaber (1960), finding that both ambenonium and methoxyambenonium antagonized the paralysis of curare and depolarizers, arrived at the conclusion that larger doses than those required to antagonize curare are needed

to restore transmission from paralysis by decamethonium. Blaber also found at these higher doses, oxamides produced a neuromuscular block resembling in many ways that caused by curare. Accordingly, he suggested that the antagonism of depolarizers was due to the curaremimetic properties of the oxamides.

The complexity of the detailed mechanism of action of oxamides is apparent; there is no correlation between the relative potency of these agents to antagonize curare paralysis and their cholinesterase inhibitor potency in vitro and while the neuromuscular block by oxamides resembles that by curare in that it is antagonized by tetanic stimulation and depolarizing agents, it is unlike that produced by curare in that it is not antagonized by neostigmine or edrophonium (Blaber, 1960).

D. DFP

Anticholinesterases, such as neostigmine and edrophonium, inhibit the enzyme "reversibly" by combining with it and blocking attachment of the substrate. Careful kinetic studies have since shown that only negligible amounts of an inhibitor of this type are released reversibly from the enzyme; indeed, neostigmine is hydrolyzed extremely slowly by cholinesterase (Wilson, 1955).

The concentrations of organophosphorus compounds which produce an effect similar to those of substances such as eserine or neostigmine are much more persistent and are not reversed by washing the preparation

(Koster, 1946). With the isopropyl group on the enzyme virtually no hydrolysis occurs and the return of cholinesterase activity depends on the formation of a new enzyme (Wilson and Bergmann, 1950). Their action is often described as "irreversible," but this is slightly misleading. Augustinsson and Nachmansohn (1949) observed that the inhibition of cholinesterases by DFP was not competitive; the reaction can be regarded as being roughly a non-competitive one. This only implies, however, that the stability of the enzyme-substrate complex is so great that it is unlikely to be broken up in vivo. The affinities of the organophosphorus compounds for the active center are not very different from those of the "reversible" inhibitors, such as eserine or neostigmine (Davison, 1955; Burgen and Hobbiger, 1951; Hobbiger, 1954; Tammelin, 1958). It is apparent that the terms "reversible" and "irreversible" as applied to these two types of anticholinesterases reflect only quantitative differences, and that both classes of drugs react with the enzyme in essentially the same manner as does the natural substrate.

DFP (diisopropyl phosphorofluoridate) was synthesized by McCombie and Saunders in 1946 and is considered an "irreversible" anticholinesterase. After DFP treatment recovery of blood and tissue cholinesterase depends on alkyl radicals and new enzyme synthesis (Blaber and Creasey, 1960a, b; Koenig and Koelle, 1961). Recovery of erythrocyte cholinesterase is 4.4% per day for the first 15 days and thereafter it is decreased to approximately 0.8% per day (Oberst and Christensen, 1956). Studies

indicate that 50 to 80% of the total cholinesterase of nervous tissue or effector organs must be inactivated before pharmacological actions are noted (Koelle and Gilman, 1946; Riker and Wescoe, 1949; Kamiyo and Koelle, 1952). Cause of death in DFP poisoning is respiratory failure and in this regard it has been shown that impairment of neuromuscular transmission occurs first, with failure of the central mechanism immediately following (Wright, 1954).

Koelle (1950, 1951, 1955, 1957) has used DFP for the purpose of determining cholinesterase inhibition by so-called "reversible inhibitors." Using cytological localization of the enzyme as a technique he has shown that the "reversible" compounds will protect the enzyme from attachment with an "irreversible" compound such as DFP. If the reversible compound inhibits the enzyme, it protects against DFP and upon dilution the enzyme can be localized. If the test compound does not inhibit cholinesterase it will not protect against DFP and an irreversible inhibition is obtained and the enzyme cannot be localized. In short, if a compound does not protect the enzyme against DFP it is not considered a "reversible" anticholinesterase.

III. HISTOCHEMISTRY

There are two general approaches to the cytological localization of sites of drug action: 1) to demonstrate at the cellular level the distribution of the active drug following its administration or 2) to demonstrate changes in a specific group or compound with which the drug is known to act. Because the compounds we are interested in are reported to have an anticholinesterase action we shall choose the second approach.

As an example of the cytological approach to pharmacology, the anticholinesterase agents represent a particularly favorable class of drugs. The primary mechanism of action of these agents is the inhibition or inactivation of cholinesterase. The thiocholine method of Koelle, when properly controlled, is both highly specific and extremely sensitive for the localization of cholinesterases. The exact site of cholinesterase, in relation to the structural elements of various tissues, had been a matter of uncertainty before Koelle's technique became available. Acetylthiocholine, used as the substrate in this technique, was first synthesized by Renshaw (1938) who noted that its pharmacological actions were similar to those of acetylcholine but of briefer duration. It is known that it is hydrolyzed at a more rapid rate than acetylcholine by cholinesterases, because of the weaker linkage of the -C-S- than the -C-O- bond. The present experiments will not attempt to differentiate between the different types of cholinesterases because in the area that is under study, the neuromyal junction, the amount of

acetylcholinesterase represents approximately 95% of the cholinesterase present in the area (Giacobini and Holmstedt, 1960).

IV. PURPOSE

Blaber (1963), comparing the same drugs as used in the present work, found that their ability to potentiate the muscle contraction produced by a close-arterial injection of acetylcholine was closely correlated with their in vitro anticholinesterase potency. However, there was no correlation between in vitro anticholinesterase potency and their ability to potentiate the muscle twitch elicited by the stimulation of the nerve, or with their anti-curare activity. It was concluded that some action, in addition to inhibition of cholinesterase, contributed to their facilitation at the neuromuscular junction, and it was suggested that this action may be at the prejunctional site.

The purpose of the present work is to try and provide more direct evidence on the ability of these compounds to inhibit cholinesterase at the dose levels Blaber used by observing their effect on muscle twitch, removing the muscle and staining for cholinesterase using the 1951 acetylthiocholine method of Koelle.

The first part of the study was a pilot study to determine the feasibility of the study. The pilot study was conducted in a small group of subjects and the results were used to determine the sample size for the main study. The main study was conducted in a larger group of subjects and the results were used to determine the effect of the intervention on the outcome variable. The results of the study showed that the intervention had a significant effect on the outcome variable. The effect was measured using a statistical test and the results were compared to the control group. The results showed that the intervention group had a significantly higher mean score than the control group. The results were statistically significant at the 5% level. The results of the study suggest that the intervention is effective in improving the outcome variable. The results of the study are consistent with the hypothesis that the intervention would have a positive effect on the outcome variable. The results of the study are also consistent with the findings of other studies in the field. The results of the study provide evidence for the effectiveness of the intervention and suggest that it may be a useful tool for improving the outcome variable. The results of the study are also consistent with the findings of other studies in the field. The results of the study provide evidence for the effectiveness of the intervention and suggest that it may be a useful tool for improving the outcome variable.

MATERIALS AND METHODS

The study was conducted in a randomized controlled trial design. The subjects were recruited from a local community and were randomly assigned to either the intervention group or the control group. The intervention group received the intervention and the control group received a placebo. The subjects were followed up for a period of 12 weeks. The outcome variable was measured at baseline and at 12 weeks. The results of the study showed that the intervention group had a significantly higher mean score than the control group at 12 weeks. The results were statistically significant at the 5% level. The results of the study suggest that the intervention is effective in improving the outcome variable. The results of the study are consistent with the hypothesis that the intervention would have a positive effect on the outcome variable. The results of the study are also consistent with the findings of other studies in the field. The results of the study provide evidence for the effectiveness of the intervention and suggest that it may be a useful tool for improving the outcome variable. The results of the study are also consistent with the findings of other studies in the field. The results of the study provide evidence for the effectiveness of the intervention and suggest that it may be a useful tool for improving the outcome variable.

I. IN VIVO PREPARATION

Forty-two adult cats were used in these experiments ranging in weight from 2.0 to 3.5 kg. The animals were anesthetized with choloralose (80 mg/kg) intraperitoneally, fastened to the operating table in a supine position and the trachea cannulated. A polyethylene cannula was placed in the left femoral artery and connected to a Stratham pressure transducer for the purpose of recording blood pressure. To prevent clotting this cannula was filled with heparin solution (200 units per ml.). Blood pressure was recorded to provide an index of the animal's condition during the experiment.

A. Preparation For The Administration Of Drugs

The left femoral vein was cannulated for intravenous drug administration. The polyethylene cannula was connected to a three-way stopcock and clamped to a support of the operating table. Since both left femoral vessels were cannulated, the left anterior tibialis muscle received none of the drugs administered and acted as a control muscle. In experiments requiring close arterial injection of the test drugs, Blaber's (1960) modification of Brown's (1938) method was used. The artery to the anterior tibialis muscle was cannulated and the polyethylene cannula filled with heparin solution (100 units per ml.), plugged until ready for use and fastened to a support of the operating table. The right femoral artery and vein were exposed and a string passed around the artery. For close arterial injections the femoral artery was occluded as the drug was injected thereby assuring the drug did pass

into the tibialis muscle. In all experiments the string was used to ligate both the femoral artery and vein shortly after all drugs had been administered.

B. Recording Of Mechanical Response

The right anterior tibialis muscle was exposed from mid-calf level to its tendon insertion. The tendon was ligatured and cut from the bone of its insertion. The sciatic nerve on the right side was exposed and cut high in the thigh. This was for the purpose of eliminating reflex responses during muscle twitch recording. The nerve to the tibialis was then exposed on the outer border of that muscle. Drills were inserted into the lower end of the femur and the lower end of the fibula and tibia and the limb was rigidly clamped in a horizontal position to the operating table. The ligature around the tendon of the muscle was connected to a Grass Model FT-10 force displacement transducer for the purpose of recording mechanical twitch. A shielded platinum electrode was placed on the tibial nerve distal to the severed sciatic nerve and connected to a Bioelectric Instrument isolator which received pulses from a stimulator consisting of a Textronix Type 162 waveform generator and a Type 161 pulse generator. The cathode of the electrode was placed nearest the muscle and a rectangular pulse of 50 micro seconds duration and twice the strength needed for maximal stimulation was used at a frequency of once every 10 seconds. A temperature probe was placed in the belly of the muscle and the muscle covered with a surgical sponge moistened with mineral oil. The muscle was maintained at 37°C

with heat lamps.

C. Experimental Procedure

The blood pressure, muscle twitch, and temperature were all monitored on an Offner Type R Dynograph. There was a 30 minute control period of muscle twitches prior to the injection of drugs. After the control period a reversible facilitatory agent was given. The drugs were given either 250 micrograms intravenously or 5 micrograms (total dose) close-arterially. All drugs injected, including DFP, were dissolved in 0.9% saline solution. Saline was also used to wash intravenous injections into the femoral venous cannula. Close-arterial injections to the anterior tibialis were administered retrogradely into the central end of the ligated tibial artery below the anterior tibialis muscle. At the moment of injection the femoral artery was occluded. The ligature around the femoral artery was released following the injection of the drug and the cannula was washed through with heparin solution (100 units per ml. in ^{0.9%} NaCl). DFP was always given intravenously (15 mg/kg). The DFP was dissolved in 0.9% saline in a concentration of 10 mg/ml and the solution prepared within one hour of its injection. In the case of those drugs which augmented the muscle twitch, the 15 mg/kg dose of DFP was injected at the point of greatest augmentation and if there was no increase in muscle twitch the DFP was given intravenously 30 seconds after the reversible facilitatory agent had been injected. At the peak of augmentation of the muscle twitch caused by DFP the right femoral artery and vein were tied off

and the stimulus to the anterior tibialis stopped. (This procedure was to make certain the drugs were not completely washed out of the muscle into the circulation). The muscle was then maintained at 36-38°C for one hour. After the one hour incubation period of the muscle in situ it was removed, immediately placed in a glass container, covered and placed in the freezer compartment of the refrigerator. The animals which survived the organophosphate toxicity were sacrificed by the injection of 30 cc of air into the femoral vein. The left anterior tibialis muscle was removed and frozen in the same manner as the right muscle.

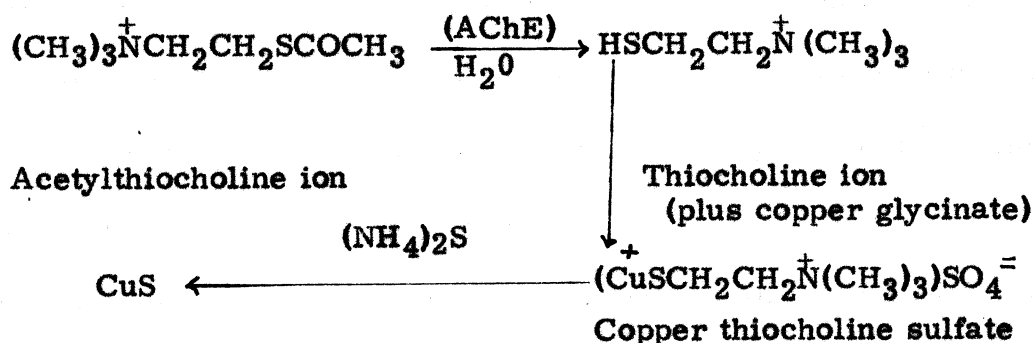
D. Histochemistry

a. Preparation of Sections

After the muscle was completely frozen a trapezoidal cross-section of the distal one-half of the muscle was removed and placed on a microtome planchet. A few drops of water were added to the planchet and the planchet with the muscle on it was then placed into the freezing microtome so that the muscle would become fixed to the planchet. The muscle was then sectioned in the microtome in 10-15 micron sections and placed on glass slides which had previously been frozen in the microtome. The slides with the muscle sections on them were placed at room temperature until thawing caused adherence of the muscle sections to the glass. This usually was 15 to 30 seconds. They were then ready to be placed in the incubation solution.

b. Staining Procedure

The histochemical approach for the determination of anticholinesterase potency in the present study is based on Koster's (1946) observation that a reversible inhibitor can prevent the lethal action of DFP by transient competitive combination with the enzyme (Koelle, 1946). The technique outlined below is Koelle's 1951 method. As developed originally (Koelle and Friedenwald, 1949), it consists in incubating fresh frozen sections in a medium containing acetylthiocholine and copper glycinate; as the substrate is hydrolyzed by acetylcholinesterase the thiocholine liberated is precipitated as a mercaptide salt, e. g., copper thiocholine sulfate. The initial concentration of the substrate (acetylthiocholine) of 5×10^{-3} M, is the optimal concentration of acetylcholine for specific cholinesterase (Augustinsson, 1949). Immersion in ammonium sulfide converts the white precipitate of copper thiocholine to a dark brown precipitate of copper sulfide which can then be viewed with an ordinary microscope.



REAGENTS

1. Copper-Glycine: 3.75 gm. glycine, 2.50 gm. copper sulfate q. s. 100 mls.
2. Maleate Buffer: 9.60 gm. sodium maleate, 52.2 mls. sodium hydroxide, q. s. 100.0 mls.
3. Sodium Sulfate: 40 per cent sodium sulfate (W/V), adjusted to pH 6.00, stored at 38°C.
4. Magnesium Chloride: 9.52 gm. magnesium chloride, q. s. 100.0 mls.
5. Acetylthiocholine: 46 mgm. acetylthiocholine iodide, 2.4 mls. water, 0.8 ml. 0.1 M copper sulfate; centrifuged; supernatant decanted off and saved.
6. Copperthiocholine: Obtained by filtering incubation solutions immediately after removal of slides, storing at 38°C. for two to four days to permit spontaneous hydrolysis of substrate, collecting precipitate by centrifugation and washing with water.
7. Incubation Solution: 0.6 ml. solution 1, 0.6 ml. water, 1.5 mls. solution 2, 10.5 mls. solution 3, 0.6 ml. solution 4, 1.2 mls. solution 5, trace of solution 6.
8. Ammonium Sulfide: 25 mls. absolute alcohol, 4.5 mls. ammonium hydroxide, 3 mls. water; saturated with hydrogen sulfide; tested for saturation with 1 drop 0.1 M copper sulfate; filtered just prior to use.

All slides were marked with a diamond pencil for identification and placed in the incubation solution for a period of 20 minutes. They were then transferred to a rinse solution of 20% sodium sulfate for 5 minutes and then immersed for 2 minutes in 10% sodium sulfate. This was followed by dipping them in distilled water for 30 seconds and transferring them to the ammonium sulfide developing solution for a period of 2 minutes. They were then stained and dehydrated by one minute rinses in alcohol and eosin, alcohol, xylol, and another xylol solution. All the above solutions were saturated with the reaction product by adding a trace of copperthiocholine. The saturation of the substrate solution with the reaction product has the advantage that diffusion of reaction product is greatly diminished or suppressed. In order to minimize spontaneous hydrolysis of the substrate (acetylthiocholine) it was added immediately before the incubation solution was to be employed. The solution was stirred and filtered through Whatman #1 filter paper into Coplin jars previously warmed in the incubator.

After the final xylol rinse the slides were mounted with Permount (Fisher) and a cover slip. Slides were then viewed under a microscope at 80X and photographed. All photomicrographs were taken with the same intensity of lighting, time of exposure, and not retouched.

II. IN VITRO PREPARATION

Adult cats were anesthetized intraperitoneally with chloralose (80 mg/kg). The right anterior tibialis muscle was removed from the animal and placed in a glass container, covered and frozen in the freezer compartment of a refrigerator. After the muscle was completely frozen, a trapezoidal cross-section of the distal one-half of the muscle was removed and placed on a microtome planchet. A few drops of water were added to the planchet and both the planchet and muscle were then placed in a freezing microtome in order that the muscle would be fixed to the planchet. When this had taken place ten-fifteen micron fresh frozen sections of the muscle were cut on the microtome and placed on slides. As soon as the sections had thawed and the excess moisture evaporated the slides were placed for 5 minutes in a Coplin jar containing a solution of the following composition: 24% sodium sulfate, 0.033 M magnesium chloride, and one of the reversible facilitatory drugs in a molar concentration calculated to be that which was in the animal's extracellular fluid following its injection in the in vivo preparation (assuming that a cat's extracellular fluid represents 15% of its body weight). The reversible inhibitor was omitted from two sets of controls. All slides excepting one set of controls were then transferred to a solution which contained the same components as above plus 10^{-4} M DFP. The slides were incubated in this solution for 30 minutes at 38° C. and then placed in a rinse solution (24% sodium sulfate, 0.033 M magnesium chloride) in which

they were kept for 30 minutes at room temperature. This latter step was repeated. All slides (including the first set of controls) were then ready to be placed in the Koelle incubation solution and to follow the same procedure as the in vivo muscle slides.

RESULTS

Before the main experiments could be carried out it was necessary to find the in vivo concentration of DFP which would consistently inhibit cholinesterase to such an extent that no staining of the enzyme could be demonstrated. The in vitro concentrations which have been used in this method for inhibition of specific esterases range from 1×10^{-1} M (Koelle, 1951) to 1×10^{-4} M (Koelle, 1957). We would expect the in vivo concentration to be similar. Using increasing doses of DFP it was found that 15 mg/kg i. v. was the lowest dose of the organophosphate which would consistently inhibit staining of cholinesterase (fig. 3). Assuming the extracellular fluid to be 15% of the body weight the in vivo molar concentration of DFP in the extracellular fluid was 1×10^{-4} M.

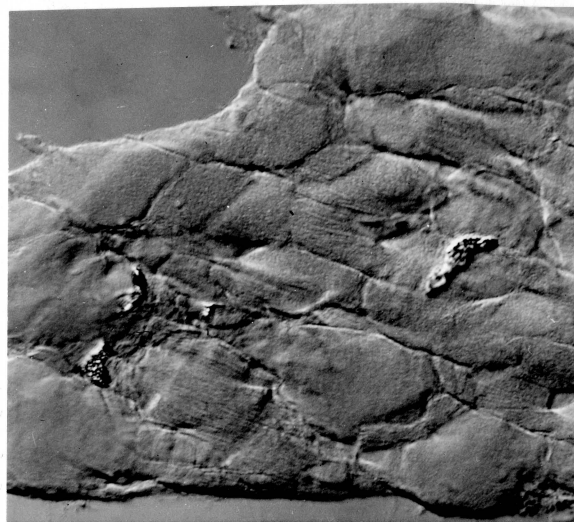
The histochemical approach to determination of the anticholinesterase actions of ambenonium, neostigmine, edrophonium and methoxyambenonium is based on the observation that the reversible inhibitor, physostigmine, can protect cats from the lethal action of the irreversible anticholinesterase agent DFP (Koster, 1946) by transient competitive combination with the enzyme (Koelle, 1946; Koelle and Koelle, 1959). Therefore, the reversible inhibitor was given immediately before a dose of DFP sufficient, if given alone, to cause practically complete inactivation of the acetylcholinesterase of a given tissue. The excess DFP and the reversible inhibitor was then subsequently removed; any residual acetylcholinesterase activity should represent that which was inhibited by the reversible inhibitor at the time the DFP was given.

FIGURE 3
CONTROLS

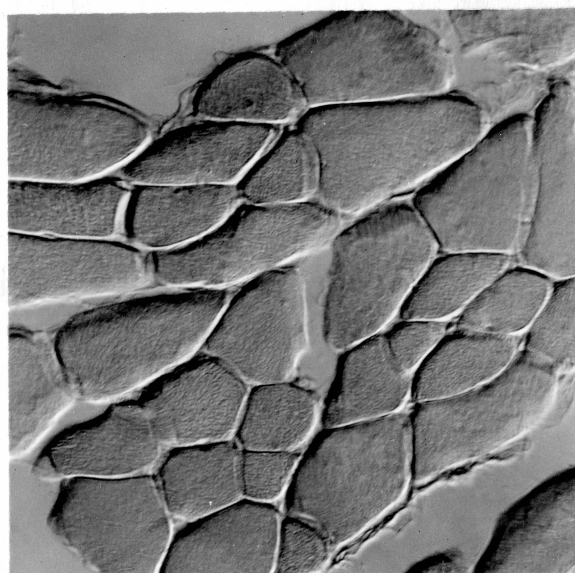
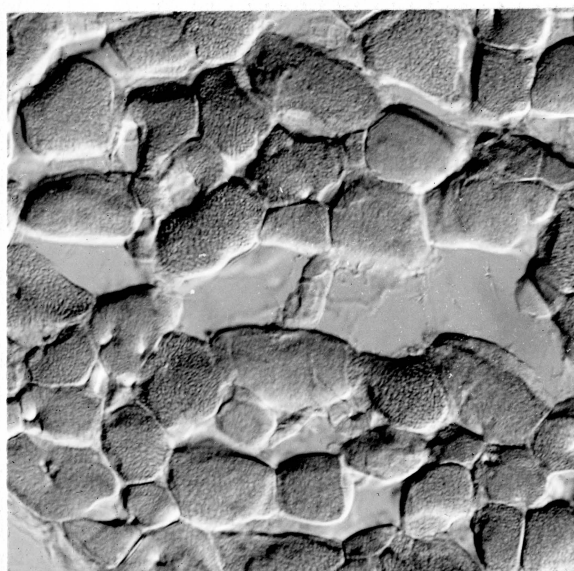
In Vivo



In Vitro



NORMAL



DFP TREATED

In the following results inhibition of muscle cholinesterase by DFP is indicated by absence of staining in the photomicrographs and shows that the test drug did not protect the enzyme from DFP and was therefore not inhibiting cholinesterase at the time the DFP was injected. Staining of the end-plates in the photographs indicates that the test drug was protecting or inhibiting the enzyme at the time the DFP was injected and reversed during the staining procedure.

I. IN VIVO

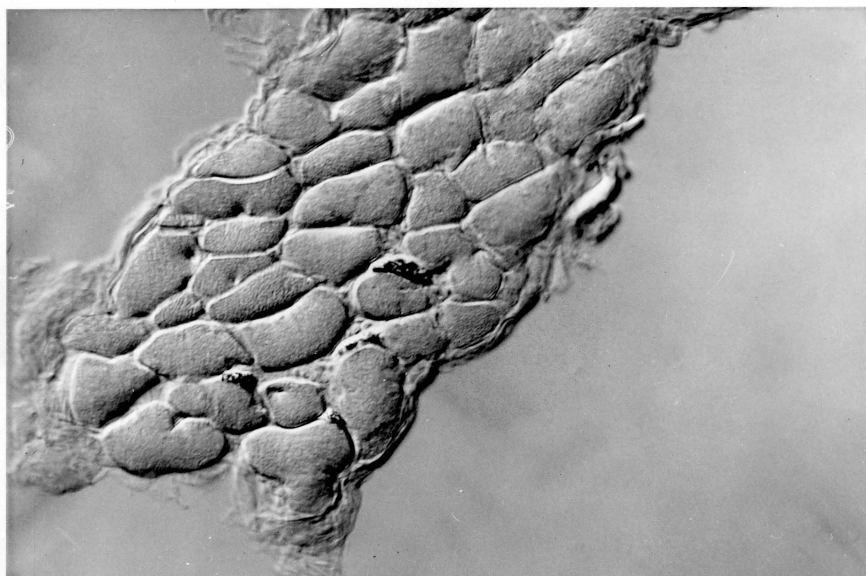
A. Neostigmine

The results with this compound are shown in fig. 4. The photomicrographs show that at the close-arterial dose there is no staining of the enzyme. We can take this to mean that neostigmine did not inhibit the enzyme prior to DFP treatment so that the organophosphate gained access to it. The photomicrograph of the intravenous dose shows staining so that neostigmine was protecting the enzyme from DFP in this situation. The muscle twitch was facilitated with both concentrations of neostigmine to the same extent and that was augmented further upon the addition of DFP. The animals receiving the low dose of neostigmine died during the incubation period with all the signs of organophosphate poisoning. The animals receiving the higher dose of neostigmine did not die after DFP although many of the signs of poisoning were apparent.

B. Edrophonium

The results of cholinesterase staining with this compound are shown in fig. 5. Neither close-arterial or intravenous doses protect against DFP. The muscle twitch was facilitated to the same extent with both concentrations and was further increased after administration of DFP. All animals died during the incubation period in vivo showing insufficient protection of the enzyme against DFP by edrophonium. The increase in twitch with both concentrations of edrophonium before the DFP was administered was in

NEOSTIGMINE IN VIVO



250 ug. i. v.



5 ug. c. a.

EDROPHONIUM IN VIVO



250 ug. i. v.



5 ug. c. a.

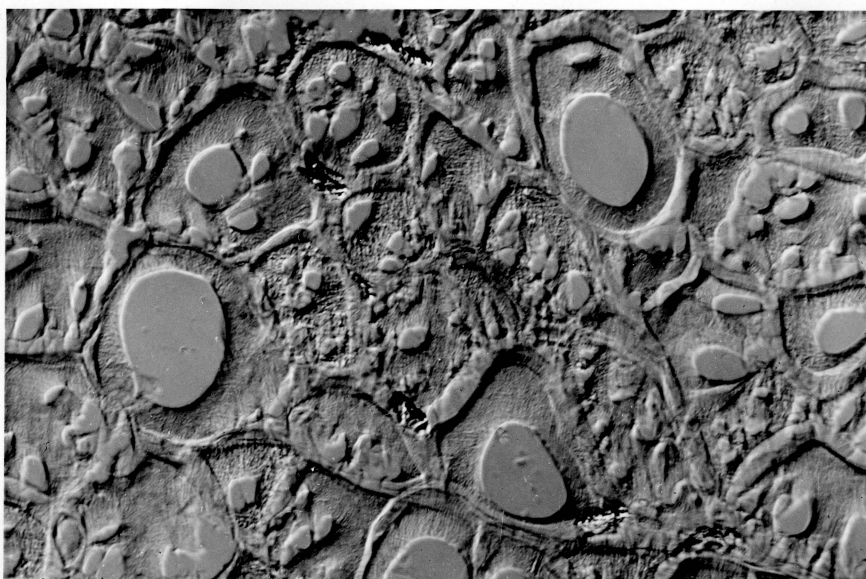
42

absence of any sign of fasciculations or other anticholinesterase intoxication signs. This has been observed by other workers (Blaber and Bowman, 1959).

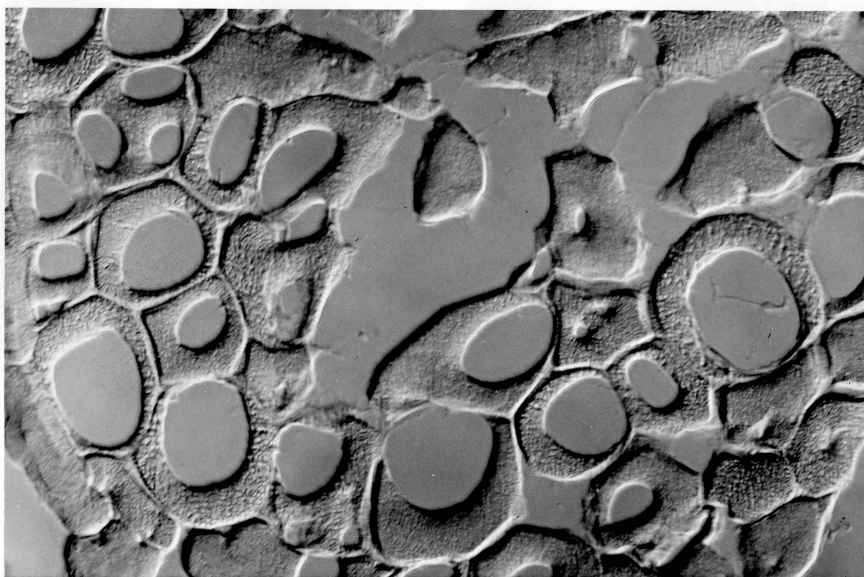
C. Methoxyambenonium

The staining produced by this compound differed from the rest since it was in such a small amount when it was observed. Since the Koelle method is mainly qualitative the results were planned to be either plus or minus with little hope of any quantitation or "partial staining." However, as shown in fig. 6 there is definitely only partial staining. This appears as uniform "hairlike" staining of acetylcholinesterase around the endplate. This type of staining indicates that methoxyambenonium does not protect the enzyme as well as neostigmine or ambenonium. The smaller dose of methoxyambenonium shows no protection at all against the action of DFP. This is in agreement with other workers who have found this compound to be a weak anticholinesterase (Karczmar and Howard, 1955; Koelle, 1957; Blaber, 1960). It was also noted with this compound that there was an increase in muscle twitch in four out of six experiments using the larger dose of the drug, which is contrary to results of other workers (Karczmar, 1957; Blaber, 1960). In one case with methoxyambenonium the animal died although partial staining of acetylcholinesterase occurred. The maximal twitch was not potentiated in this case. In all cases the animals were in poor condition during the in vivo incubation period of the muscle with all but one of the animals dying of those given the low dose of methoxyambenonium.

METHOXYAMBENONIUM IN VIVO



250 ug. i. v.

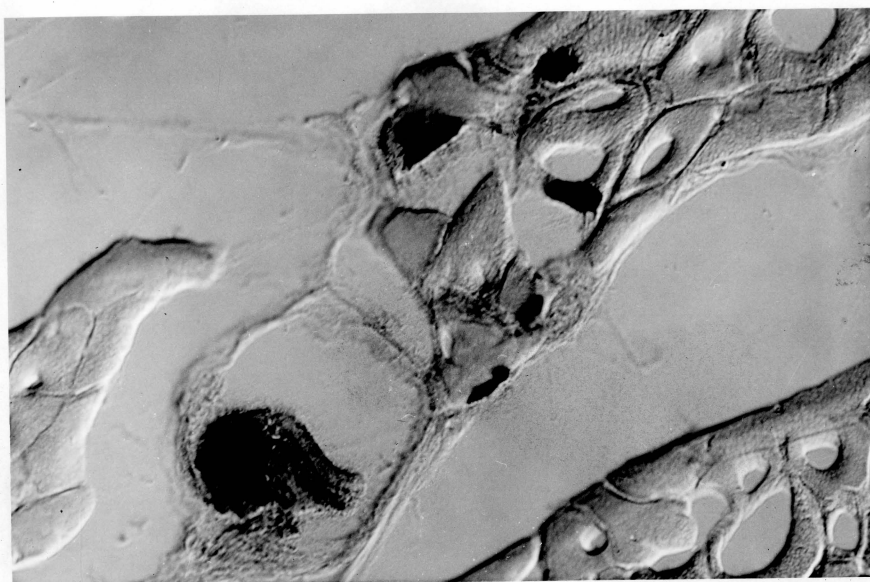


5 ug. c. a.

D. Ambenonium

Figure 7 shows that this compound stains well with the intravenous or close-arterial dose of the drug. Staining of cholinesterase when this compound is used to protect the enzyme against DFP is expected at even lower doses than used here (Koelle, 1957). There is evidence from these photographs that the amount of staining is greater here than it was in the control sections. The increase in twitch with both doses was greater compared to that obtained with all other facilitators. This facilitation was such that the injection of DFP did not increase it further. There were fasciculations and other signs similar to those seen in the course of organophosphate poisoning before the DFP was given; however, none of the animals died after DFP, so the compound seemed capable of protecting against the lethal affects of DFP.

FIGURE 7
AMBENONIUM IN VIVO



250 ug. i. v.



5 ug. c. a.

II. IN VITRO

A. Neostigmine

The results in vitro with this compound are shown in fig. 8. These pictures mimic those of the in vivo results with this compound. There appears to be some diffusion that was not apparent in vivo. However, the low concentration (3.32×10^{-8} M) of neostigmine shows no staining as before and, likewise, the higher concentration (1.65×10^{-6} M) shows staining.

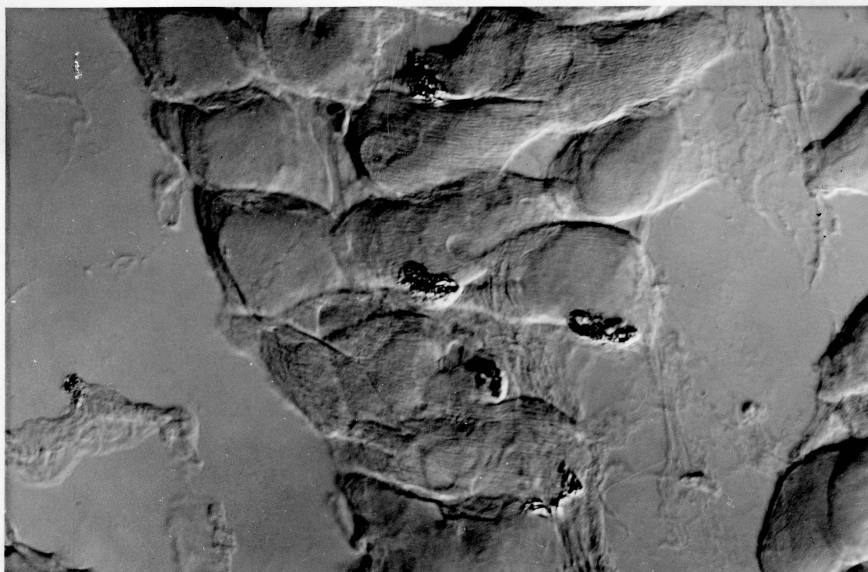
B. Edrophonium

Figure 9 show the results with edrophonium. These are the same as those found in vivo showing no staining at either the low (5.52×10^{-8} M) or high (2.76×10^{-6} M) concentration. Unless concentrations of 1×10^{-3} M edrophonium are used with this technique the end-plates are not expected to stain (Christoff, Anderson, Slotwiner, and Song, 1966).

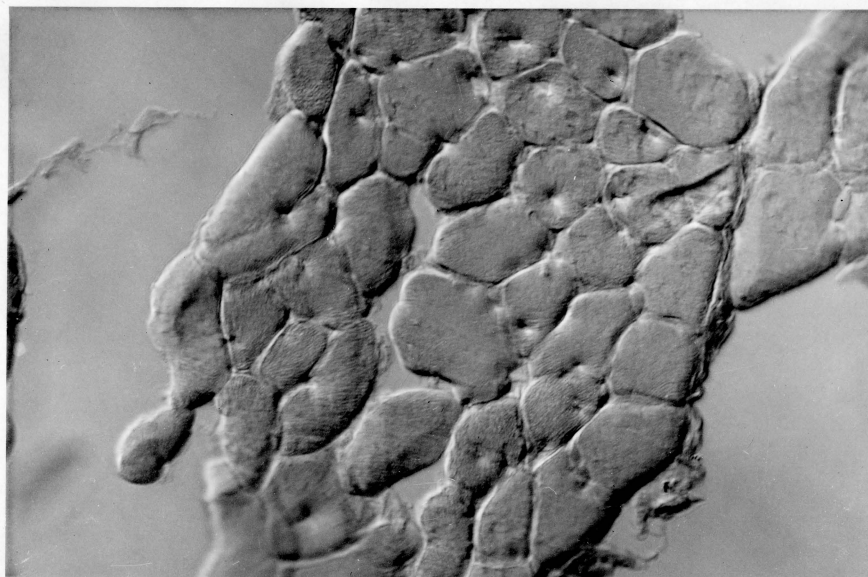
C. Methoxyambenonium

The results with methoxyambenonium are shown in fig. 10. These were not the same as those seen in vivo. With the higher concentration (9.25×10^{-7} M) we see the "hairlike" partial staining which we assume is partial protection of the enzyme by the compound. Here, however, this type of staining occurred also at the lower concentration (1.85×10^{-8} M). The amount of staining seen in the sections does not appear to differ.

NEOSTIGMINE IN VITRO



$1.65 \times 10^{-6} M$



$3.32 \times 10^{-8} M$

FIGURE 9

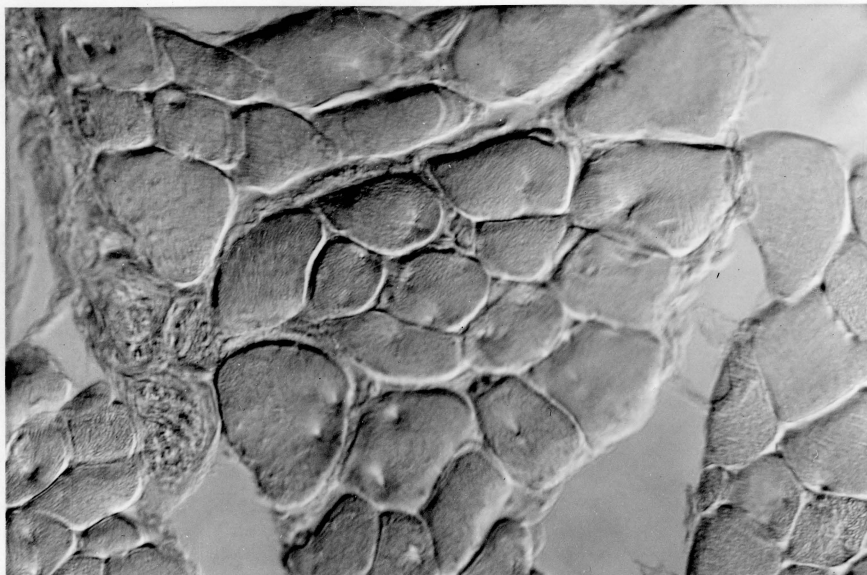
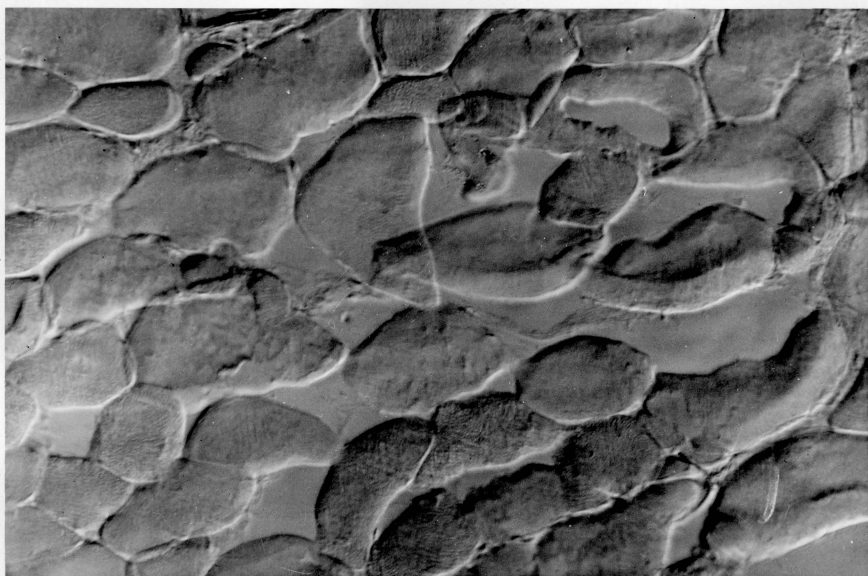
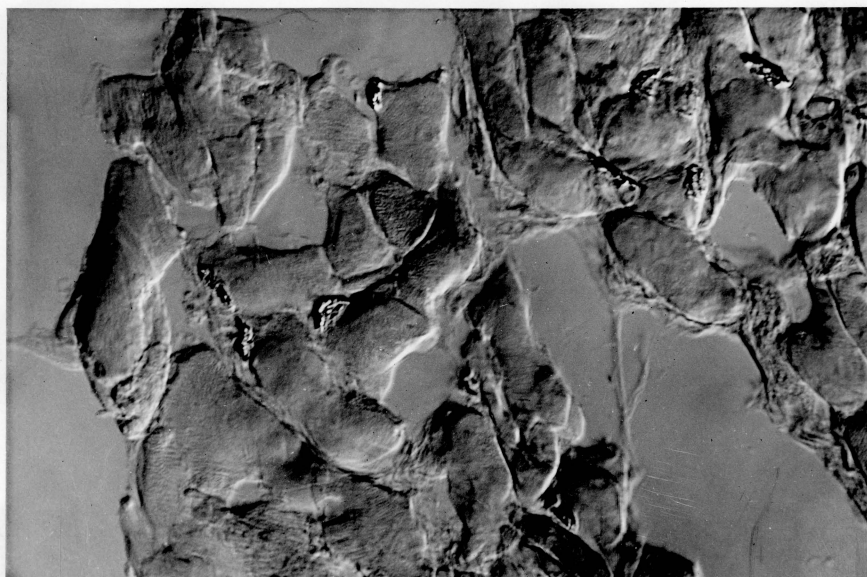
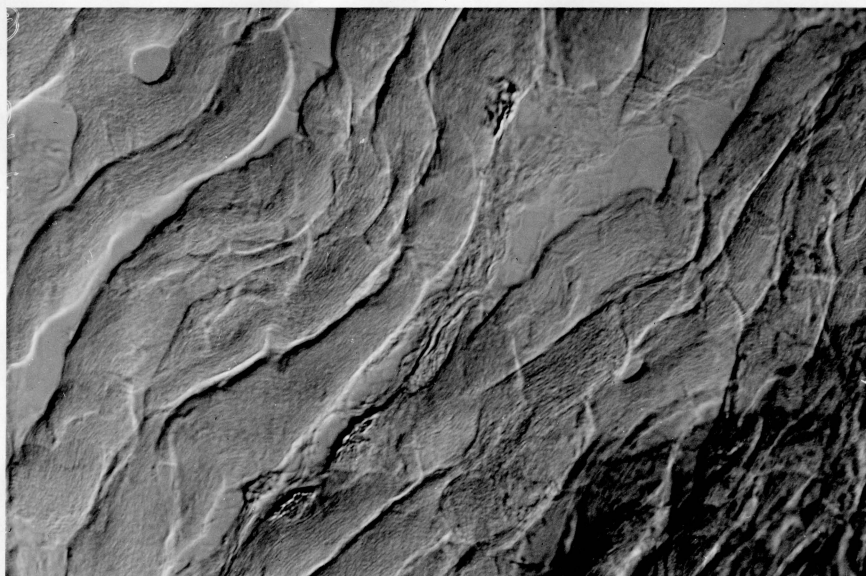
EDROPHONIUM IN VITRO $2.76 \times 10^{-6} \text{M}$  $5.52 \times 10^{-8} \text{M}$

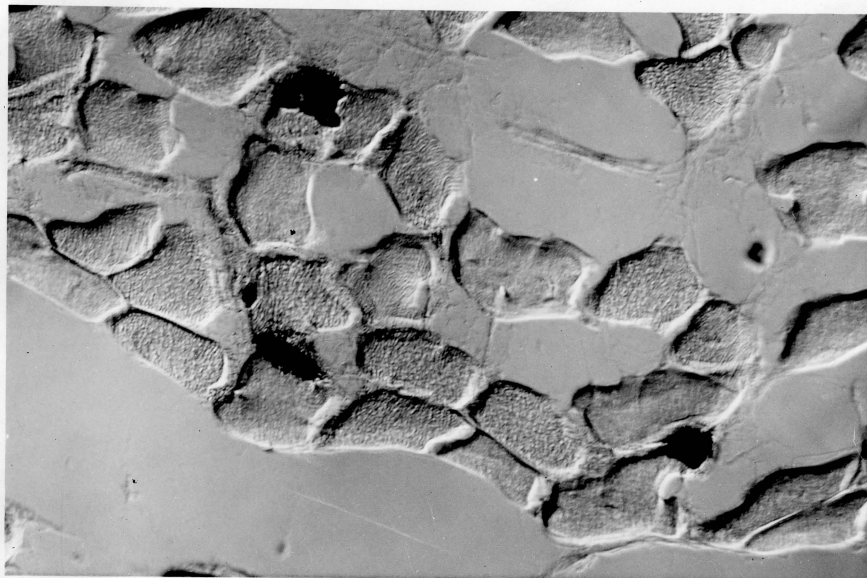
FIGURE 10

METHOXYAMBENONIUM IN VITRO $9.25 \times 10^{-7} \text{M}$  $1.35 \times 10^{-8} \text{M}$

D. Ambenonium

With this compound there was staining at both the low ($1.83 \times 10^{-8}M$) and high ($9.15 \times 10^{-7}M$) concentrations. Figure 11 indicates a more intense stain than that of the control sections so that these results are in agreement with those obtained in vivo. The in vivo and in vitro results with this compound show a much greater amount of stain occurring with it than with the methoxy derivative. This would agree with others who have found it to be 100 times as potent an inhibitor of cholinesterase as methoxyambenonium (Koelle, 1957; Elaber, 1960).

AMBENONIUM IN VITRO



$9.15 \times 10^{-7} \text{M}$



$1.83 \times 10^{-8} \text{M}$

DISCUSSION

The results presented in this thesis (fig. 12) have demonstrated a lack of correlation between the abilities of certain facilitatory drugs to potentiate the maximal indirect twitch and to inhibit the acetylcholinesterase present in skeletal muscle. These results therefore support the conclusions of others (Randall, 1950; Wescoe and Riker, 1951; Riker, 1953; Karczmar, 1957; Blaber, 1960; Blaber and Bowman, 1962; Blaber, 1963 a, b) that some action or actions, in addition to cholinesterase inhibition, play an important part in the ability of these drugs to potentiate the responses of the muscle to nerve stimulation.

In the present experiments ambenonium proved to be the most potent anticholinesterase of the drugs used. Following the injection of this compound the amount of cholinesterase staining, the increase in the size of muscle twitch, and the fasciculations observed in the animals all pointed to cholinesterase inhibition. It also proved capable of protecting the animal against the lethal dose of DFP even though it produced signs similar to organophosphate poisoning. This compound certainly may cause facilitation at the neuromuscular junction through an anticholinesterase action.

Edrophonium was not inhibiting cholinesterase at the dose levels used in the experiments, or it was rapidly disassociated in the presence of DFP in a similar way to that described by Wilson (1955) in the presence of acetylcholine. It has been shown recently that concentrations of the order of 1×10^{-3} M of edrophonium are necessary with this technique before

FIGURE 12
SUMMARY OF RESULTS

ACHE INHIBITION

DRUG	INVIVO		INVITRO	
	250 μ g iv.	5 μ g c.a.	Low Conc.	High Conc.
NEOSTIGMINE	+	—	—	+
EDROPHONIUM	—	—	—	—
AMBENONIUM	+	+	+	+
METHOXY- AMBENONIUM	+ —	—	+ —	+ —

staining of cholinesterase will appear (Christoff, Anderson, Slotwimer and Song, 1966). In microelectrode studies anticholinesterase effects are seen with edrophonium, but at concentrations higher than the lower dose used in the present experiments which caused an increase in muscle twitch (Christ, personal communication). The lack of fasciculations or other anticholinesterase signs following the administration of edrophonium and before the DFP was given coupled with the absence of histochemical staining would substantiate a theory that the increase in muscle twitch with edrophonium may be caused via a mechanism other than cholinesterase inhibition.

In the present studies the low concentration of methoxyambenonium produced some protection in vitro but generally not in vivo. The high concentration also appeared to produce only partial protection. This may indicate that the association of the drug with the cholinesterase molecule is slow, and at the low concentration in vivo there was insufficient time for any appreciable inhibition. The facilitation of muscle twitch was immediate, and the anticurare effect of the compound has also been reported to be rapid (Blaber, 1960). The total absence of any observable anticholinesterase signs in the animal preceding the injection of DFP coupled with the anticurare potency of this compound would certainly imply a mechanism of action for this compound other than cholinesterase inhibition. The slight staining of the enzyme in the present experiments proved to be inadequate to cause any signs of cholinesterase inhibition that we would expect and was unable to

protect the animal from the DFP. This may mean that the cholinesterase molecule is a "secondary" or "tertiary receptor" for this compound. That is, only at higher dose levels than used in the present experiments would the anticholinesterase action of this compound possibly contribute significantly to its effects. Compounds which have an effect at the neuromuscular junction would be expected to fit many receptors in that area, but each receptor's attraction to a particular compound is different. The fact that methoxyambenonium is very slow to dissociate from cholinesterase (Koelle, 1957) may account for the compound possessing little anticholinesterase potency and yet still remain on the molecule throughout the DFP treatment and staining procedure.

In low doses neostigmine gave gross and histochemical signs similar to those obtained with edrophonium, i. e., an increase in muscle twitch without gross anticholinesterase signs in the animal or any histochemical staining of cholinesterase, and the animal dying after DFP treatment. With an increase in dose, neostigmine gave results like those obtained with ambenonium. There were fasciculations in the animal, a large increase in muscle twitch, protection against the lethal effects of DFP, and histochemical staining of cholinesterase. It would therefore seem that neostigmine in lower doses exerts its action on a receptor other than the cholinesterase molecule. At higher dose levels cholinesterase inhibition will undoubtedly add to the action, but to what extent this is important in the facilitation of

neuromuscular transmission is debatable. One should only assume that it is able to inhibit cholinesterase at higher dose levels which may not add to its action.

One cannot quantitate the staining in the muscle sections. There is little information in the literature as to how much cholinesterase there has to be present to stain. One can compare grossly the amount of staining between muscle sections though, and there is no doubt in the present studies that methoxyambenonium treated muscle was intermediate in staining between edrophonium and ambenonium.

The efficacy of the method used in promoting immediate precipitation is, of course, dependent upon the adequate penetration of both copper thiocholine and copper glycinate from the surrounding medium to the sites of the enzyme in the tissues. The intensity of the specific stain at any site is dependent upon many factors, foremost of which are probably the concentration of the enzyme and its reaction velocity or turnover number. The localization of specific cholinesterase is favored in the present technique by a substrate concentration (5×10^{-3} M) in the optimal range for the enzyme (Augustinsson, 1948). However, nonspecific esterases can also hydrolyze the substrate at this concentration, although at a much slower rate. In any histochemical procedure the question arises as to whether or not the area where a precipitate is seen represents the actual site of enzymatic action. The alternative interpretation is that diffusion of the

initial product of hydrolysis or of the subsequent reaction product has occurred, followed by a precipitation at an adjacent site. With the present method the possibility of diffusion prior to precipitation was minimized by the addition of concentrated sodium sulfate and a pH of 6.1 (Koelle, 1951).

Since the results of the present experiments do not agree with the classical anticholinesterase action of the compounds used, an alternate theory is needed to explain their effects.

An action of acetylcholine at the autonomic ganglia, which may also operate at the neuromuscular junction, has been suggested by Volle and Koelle (1961) to be part of normal synaptic transmission. According to their hypothesis, the pre-synaptic nerve impulse causes the release of a small amount of acetylcholine which then acts on the pre-synaptic nerve endings to cause a greater release of the same transmitter which is then sufficient to depolarize the post-synaptic receptor. They have also proposed that an important function of ganglionic acetylcholinesterase may be protection of the preganglionic terminations from depolarization and antidromic firing in response to acetylcholine liberated by themselves and adjacent terminations. Whether or not this happens at the neuromuscular junction has not been shown. Hubbard, Schmidt and Yokota, (1965), have provided direct evidence for a depolarizing action of acetylcholine on nerve terminals. It appears that the physiological action of acetylcholine may provide a negative feedback on further acetylcholine release. However,

59
Koelle (personal communication) is describing events that occur during one action potential, whereas Hubbard, Schmidt and Yokota are discussing the effects of successive action potentials on each other. This provides another cholinceptive site for drug action at the neuromuscular junction. A mechanism for neuromuscular facilitation using anticholinesterases would be inhibition of cholinesterase allowing acetylcholine to work at the pre-synaptic site (Eccles, Katz, Kuffler, 1942). The results of the present experiments indicate that ambenonium could work via this mechanism. The other compounds did not inhibit cholinesterase, and if they facilitate transmission via a presynaptic site it must be through a direct action at the receptor site.

Karczmar, in considering the actions of certain anticholinesterase inhibitors at the neuromyal junction, found that the mono- and bis-quaternary inhibitors exhibit other actions which are not dependent on acetylcholinesterase inhibition. There is incomplete correlation between acetylcholinesterase inhibition, acetylcholine twitch, curare antagonism, and muscle twitch potentiation (Karczmar, 1957). An interesting phenomenon in the frog is augmentation without prolongation of the endplate potential or acetylcholine depolarization (Karczmar, Kim, Koketsu, 1961). These workers conclude that these compounds sensitize the endplate, since they facilitate depolarization of the endplate without having a depolarizing action of their own.

Neostigmine and edrophonium possess a direct depolarizing action at the motor endplate but this action has been shown to be too weak to

contribute to the facilitating action of these drugs on neuromuscular transmission (Hobbiger, 1952; Smith, Cohen, Pelikan and Unna, 1952; Nastuk and Alexander, 1954).

Many experiments in which the effects of these drugs were tested on muscle and motor nerve action potentials led to the conclusion that they possess an action on the motor nerve endings. Twenty-six years ago it was shown that antidromic nerve activity occurred from the ventral roots of cats during the muscular contraction provoked by the intra-arterial injection of acetylcholine. It was suggested as a result of these experiments that neostigmine inhibited acetylcholinesterase and allowed acetylcholine to work on the nerve terminal (Masland and Wigton, 1940). However, it is clear from the calculations of Ogston (1955), that even if not destroyed by cholinesterase, this acetylcholine would rapidly diffuse away from the junctional region. The year following Masland's and Wigton's work, Feng and Li (1941) suggested that anticholinesterases worked directly on the nerve terminal. Other workers concluded that repetitive firing of the muscle caused the backfiring on the nerve (Eccles, Katz and Kuffler, 1942). This latter concept was accepted until Riker, Roberts, Standaert and Fujimori (1957), using a compound which blocked muscle potentials, were still able to record antidromic phenomenon on the nerve. It has been shown that the whole of the summed muscle action currents are required to produce a back response in responsive nerve fibers (Lloyd, 1942; Brown and Matthews, 1960).

23

This is the ephaptic back response initiated in some motor fibers by the muscle action current. These workers have also demonstrated that when the muscle is submaximally stimulated the ephaptic potential disappears. Anticholinesterase compounds have been observed to cause backfiring in ventral roots in the presence of a submaximally stimulated muscle (Werner, 1960). Blaber and Bowman (1963b) have shown that the duration of the repetitive discharges produced by close-arterially injected edrophonium and neostigmine was approximately 50 milliseconds following each main nerve spike, and this corresponds closely with the duration of the negative after-potential of the nerve terminal as recorded by Hubbard and Schmidt (1961). Ambenonium was observed to cause antidromic firing for 300 milliseconds and methoxyambenonium was the only drug studied which, by itself, was unable to elicit antidromic activity in motor axons (Blaber and Bowman, 1963b). From the results of the present experiments it seems that the prolonged duration of repetitive firing produced by ambenonium in Blaber's and Bowman's experiments may well have been due to preserved acetylcholine. This is not true of edrophonium and neostigmine which could act via the pre-synaptic mechanism, nor of methoxyambenonium which appears to act via a mechanism which does not involve preservation of transmitter or the pre-synaptic mechanism.

The results obtained in the present study support the view that facilitatory drugs act at more than one site at the neuromuscular junction.

All the drugs used in this work have been shown to inhibit cholinesterase to some extent (Blaber, 1963), all appear to have some action at the nerve terminal (Blaber, et al, 1963 a, b) and some have been shown to possess either a curare-like, depolarizing, or sensitizing action on the end-plate (Karczmar, 1957; Karczmar, et al, 1961). This suggests that the receptor characteristics at all three sites are similar and it may be impossible to produce a drug which selectively combines at one site only. To produce any one of these actions, different molecular characteristics are required which frequently, but not necessarily, occur in association with each other. Viewed in this way, we are merely measuring a degree of overlap of classes of agents endowed with independently variable actions. Interaction between different receptor sites must always occur, particularly with higher concentrations produced by intravenous administration, and this probably explains why their effects on transmission cannot be correlated with any single action of the drugs. Our results show that with close arterial injections facilitation of muscle twitch results, without inhibition of cholinesterase, which differed from intravenous administration of the compounds. Ambenonium was the only drug used which inhibited cholinesterase to a large extent during c. a. injection, and it is therefore possible that the action of ambenonium is more dependent upon preserved acetylcholine at effector cells than at other sites where the drug has actions.

BIBLIOGRAPHY

Abdon, N. O., and T. Bjarke: The mechanism of acetylcholine liberation in striped muscles. *Acta pharm. tox.*, Kbh. 1, 1-17 (1945).

Anderson-Cedergren, E.: Ultrastructure of motor end-plates and sarco-plasmic components of mouse skeletal muscle fiber. *J. Ultrastruct. Res.*, Suppl. 1 (1959).

Arnold, A., A. E. Soria and F. K. Kirchner: A new anticholinesterase oxamide. *Proc. Soc. Exper. Biol. (N.Y.)* 87, 393-394 (1954).

Augustinsson, K. B.: Cholinesterases; a study in comparative enzymology. *Acta physiol. scand.* 15, (Suppl. 32), 1-182 (1949).

Augustinsson, K. B., and D. Nachmansohn: Studies on cholinesterase. VI. Kinetics of the inhibition of acetylcholine esterase. *J. biol. Chem.* 179, 543-559 (1949).

Barrnett, R. J.: The fine structural localization of acetylcholinesterase at the myoneural junction, *J. Cell. Biol.* 12, 247-262, (1962).

Beckett, E. B., and G. H. Bourne: Cholinesterase in normal and abnormal human skeletal muscle. *J. Neurol. Psychiat.* 20, 191-197 (1957).

Berman-Reisberg, R.: Properties and biological significance of choline acetylase. *Yale J. biol. Med.* 29, 403-435 (1957).

Birks, R. I., and F. C. MacIntosh: Acetylcholine metabolism at nerve-endings. *Brit. med. Bull.* 13, 157-161 (1957).

Birks, R. I., and F. C. MacIntosh: Acetylcholine metabolism of a sympathetic ganglion. *Canad. J. Biochem. Physiol.* 39, 787-827 (1961).

Birks, R. I., H. E. Huxley and B. Katz: The fine structure of the neuromuscular junction of the frog. *J. Physiol. (Lond.)* 150, 134-144 (1960).

Blaber, L. C.: The antagonism of muscle relaxants by ambenonium and methoxyambenonium in the cat. *Brit. J. Pharmacol.* 15, 476-484 (1960).

Blaber, L. C.: Ph.D. thesis, University of London, London, England. (1962).

Blaber, L. C.: Facilitation of neuromuscular transmission by anticholinesterase drugs. *Brit. J. Pharmacol.* 20, 63-73 (1963).

Blaber, L. C., and W. C. Bowman: A comparison between the effects of edrophonium and choline in the skeletal muscles of the cat. *Brit. J. Pharmacol.* 14, 456-466 (1959).

Blaber, L. C., and W. C. Bowman: The interaction between benzoquinonium and anticholinesterases in skeletal muscle. *Arch. Int. Pharmacodyn.* 138, 90-104 (1962).

Blaber, L. C., and W. C. Bowman: Studies on the repetitive discharges evoked in motor nerve and skeletal muscle after injection of anticholinesterase drugs. *Brit. J. Pharmacol.* 20, 326-344 (1963a).

Blaber, L. C., and W. C. Bowman: The effects of some drugs on the repetitive discharges produced in nerve and muscle by anticholinesterases. *Int. J. Neuropharmacol.* 2, 1-16 (1963b).

Blaber, L. C., and N. H. Creasey: The mode of recovery of cholinesterase activity in vivo after organophosphorus poisoning. 1. Erythrocyte cholinesterase. *Biochem. J.* 77, 591-596 (1960a).

Blaber, L. C. and N. H. Creasey: The mode of recovery of cholinesterase activity in vivo after organophosphorus poisoning. 2. Brain cholinesterase. *Biochem. J.* 77, 597-604 (1960b).

Bowman, W. C.: Mechanisms of neuromuscular blockade. *Progress in Medicinal Chemistry*. Vol. 2. ed. G. P. Ellis and G. B. West. 88-131, London. Butterworths (1962).

Boyd, I. A., and A. R. Martin: The end-plate potential in mammalian muscle. *J. Physiol. (Lond.)* 132, 74-91 (1956).

Brooks, V. B., and D. K. Myers: Cholinesterase content of normal and denervated skeletal muscle in the guinea-pig. *J. Physiol. (Lond.)* 116, 158-167 (1952).

Brown, G. L.: Action potentials of normal mammalian muscle. Effects of acetylcholine and eserine. *J. Physiol.* 89, 220-237 (1937).

Brown, G. L.: The preparation of the tibialis anterior (cat) for close-arterial injections. J. Physiol. 92, 22P-23P (1938).

Brown, G. L., H. H. Dale and W. Feldberg: Reactions of the normal mammalian muscle to acetylcholine and to eserine. J. Physiol. 87, 394-424 (1936).

Brown, M. D., and P. B. C. Matthews: The effect on a muscle twitch of the back-response of its motor nerve fibers. J. Physiol. 150, 332-346 (1960).

Burgen, A. S. V., and F. Hobbiger: The inhibition of cholinesterases by alkylphosphates and alkyl phenol phosphates. Brit. J. Pharmacol. 6, 592-605 (1951).

Christoff, N., P. J. Anderson, P. Slotwiner, and S. K. Song: Electrophoretic and histochemical evaluation of anticholinesterase drugs. Ann. N. Y. Acad. Sci. 135, 150-163 (1966).

Clark, D. A.: Muscle counts of motor units; a study in innervation ratios. Amer. J. Physiol. 96, 296-304 (1931).

Coers, C., and A. Z. Woolf: The innervation of Muscle: A Biopsy Study. Springfield, Illinois. Charles C. Thomas 1959.

Couteaux, R.: Nouvelles observations sur la structure de la plaque motrice et interpretation des rapports myo-neuraux. C. R. Soc. Biol. (Paris) 138, 976-978 (1944).

Couteaux, R. : Contribution à l'étude de la synapse myoneurale; buisson de Kälhne et plaque motrice. Rev. canad. Biol. 6, 563-711 (1947).

Couteaux, R. : Motor end-plate structure, In "Structure and Function of Muscle." vol. 1. ed. G. H. Bourne. New York. Academic Press 1960.

Couteaux, R. : Principaux critères morphologiques et cytochimiques utilisables aujourd'hui pour définir les diverstypes de synapses. Actualités neurophysiol. 3, 145-173 (1961).

Couteaux, R., and D. Nachmansohn: Changes of cholinesterase at end-plates of voluntary muscle following section of sciatic nerve. Proc. Soc. exp. Biol. (N.Y.) 43, 177-181 (1940).

Couteaux, R., and J. Taxi: Recherches histochimiques sur la distribution des activités cholinestérasiqes au niveau de la synapse myoneurale. Arch. Anat. micr. Morph. exp. 41, 352-392 (1952).

Cowan, S. L. : The action of eserine-like and curare-like substances on the responses of frog nerve-muscle preparations to repetitive stimulation. J. Physiol. 93, 215-262 (1938).

Davison, A. N.: Return of cholinesterase activity in the rat after inhibition by organophosphorus compounds. Biochem. J. 60, 339-346 (1955).

Del Castillo, J., and L. Engbaek: The nature of the neuromuscular block produced by magnesium. *J. Physiol.* 124, 370-384 (1954).

Del Castillo, J., and B. Katz: On the localization of acetylcholine receptors. *J. Physiol.* 128, 157-181 (1955).

Denz, F. A.: On the histochemistry of the myoneural junction. *Brit. J. exp. Path.* 34, 329-339 (1953).

Eccles, J. C., B. Katz and S. W. Kuffler: Nature of the "endplate potential" in curarized muscle. *J. Neurophysiol.* 4, 362-387 (1941).

Eccles, J. C., B. Katz and S. W. Kuffler: Effect of eserine on neuromuscular transmission. *J. Neurophysiol.* 5, 211-230 (1942).

Fatt, P., and B. Katz: Spontaneous subthreshold activity at motor nerve endings. *J. Physiol.* 117, 109-128 (1952).

Feng, T. P., and Y. C. Ting: Studies on the neuromuscular junction XI. A note on the local concentration of cholinesterase at motor nerve endings. *Chin. J. Physiol.* 13, 141-143 (1938).

Feng, T. P., and T. H. Li: Studies on the neuromuscular junction XXIII. A new aspect of the phenomena of eserine potentiation and post-tetanic facilitation in mammalian muscles. *Chin. J. Physiol.* 16, 37-56 (1941).

Fukuda, R., and G. B. Koelle: The cytological localization of intracellular neuronal acetylcholinesterase. J. biophys. biochem. Cytol. 5, 433-440 (1959).

Gasser, H. S.: The classification of nerve fibers. Ohio J. Sci. 41, 145-159 (1941).

Gerebtzoff, M. A.: Cholinesterases; A Histochemical Contribution to the Solution of Some Functional Problems. New York. Pergamon Press 1959.

Giacobini, E., and B. Holmstedt: Cholinesterase in muscles: a histochemical and microgasometric study. Acta pharmacol., Kbh. 17, 94-105 (1960).

Hobbiger, F. W.: The mechanism of anticurare action of certain neostigmine analogues. Brit. J. Pharmacol. 7, 223-236 (1952).

Hobbiger, F. W.: Anticholinesterases. A comparison between the in vitro activity and the in vivo action of certain organic phosphates. Chem. and Ind. 1954, 1574 (1954).

Holmstedt, B.: A modification of the thiocholine method for determination of cholinesterase. II. Histochemical application. Acta physiol. scand. 40, 322-330 (1957).

Holmstedt, B.: Pharmacology of organophosphorus cholinesterase inhibitors. Pharm. Rev. 11, 567-688 (1959).

Hubbard, J. I.: The effect of calcium and magnesium on the spontaneous release of transmitter from mammalian motor nerve endings. J. Physiol. 159, 507-517 (1961).

Hubbard, J. I., and R. F. Schmidt: Stimulation of motor nerve terminals. Nature (Lond.) 191, 1103-1104 (1961).

Hubbard, J. I., R. F. Schmidt and T. Yokota: The effect of acetylcholine upon mammalian motor nerve terminals. J. Physiol. 181, 810-828 (1965).

Kamijo, K., and G. B. Koelle: The relationship between cholinesterase inhibition and ganglionic transmission. J. Pharmacol. exp. Ther. 105, 349-357 (1952).

Karczmar, A. G.: Antagonism between a bis-quaternary oxamide, WIN 8078, and depolarizing and competitive blocking agents. J. Pharmacol. exp. Ther. 119, 39-47 (1957).

Karczmar, A. G., and S. W. Howard: Antagonism of d-tubocurarine and other pharmacologic properties of certain bis-quaternary salts of basically substituted oxamides (WIN 8077 and analogs). J. Pharmacol. exp. Ther. 113, 30 (1955).

Karczmar, A. G., K. C. Kim and K. Koketsu: Endplate effects and antagonism to d-tubocurarine and decamethonium of tetraethylammonium and of methoxyambenonium (WIN 8078). J. Pharmacol. 134, 199-205 (1961).

Koelle, G. B.: Protection of cholinesterase against irreversible inactivation by di-isopropyl fluorophosphate (DFP) in vitro.

J. Pharmacol. exp. Ther. 88, 232-237 (1946).

Koelle, G. B.: The histochemical differentiation of types of cholinesterases and their localization in tissues of the cat. J. Pharmacol. exp. Ther. 100, 158-179 (1950).

Koelle, G. B.: The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distributions. J. Pharmacol. exp. Ther. 103, 153-171 (1951).

Koelle, G. B.: The histochemical identification of acetylcholinesterase in cholinergic, adrenergic and sensory neurons. J. Pharmacol. exp. Ther. 114, 167-184 (1955).

Koelle, G. B.: Histochemical demonstration of reversible anticholinesterase action at selective cellular sites in vivo. J. Pharmacol. exp. Ther. 120, 488-503 (1957).

Koelle, G. B., and A. Gilman: The relationship between cholinesterase inhibition and the pharmacological action of di-isopropyl fluorophosphate. J. Pharmacol. exp. Ther. 87, 421-434 (1946).

Koelle, G. B., and J. S. Friedenwald: A histochemical method for localizing cholinesterase activity. Proc. Soc. exp. Biol. (N. Y.) 70, 617-622 (1949).

Koelle, W. A. and G. B. Koelle: The localization of external or functional acetylcholinesterase at the synapses of autonomic ganglia. J. Pharmacol. 126, 1-8 (1959).

Koenig, E., and G. B. Koelle: Mode of regeneration of acetylcholinesterase in cholinergic neurons following irreversible inactivation. J. Neurochem. 8, 169-188, (1961).

Koster, R.: Synergisms and antagonisms between physostigmine and di-isopropyl fluorophosphate in cats. J. Pharmacol. exp. Ther. 88, 39-46 (1946).

Krnjević, K., and R. Miledi: Acetylcholine in mammalian neuromuscular transmission. Nature (Lond.) 182, 805-806 (1958).

Lands, A. M., and A. G. Karczmar: Mechanism of d-tubocurarine antagonism of certain bis quaternary salts of basically substituted oxamides (WIN 8077 and analogs). Fed. Proc. 14, 361 (1955).

Lloyd, D. P. C.: Stimulation of peripheral nerve terminations by active muscle. J. Neurophysiol. 5, 153-165 (1942).

Loewi, O.: "Über humorale Übertragbarkeit der herznervenwirkung. Pflügers Arch. ges. Physiol. 189, 239-242 (1921).

Loewi, O., and E. Navratil: "Über humorale Übertragbarkeit der herzenwirkung. X. Über das schicksal des vagusstoffes. Pflügers Arch. ges. Physiol. 214, 678-688 (1926).

Macfarlane, D. W., E. W. Pelikan and K. R. Unna: Evaluation of curarizing drugs in man. V. Antagonism to curarizing effects of d-tubocurarine by neostigmine, m-hydroxy phenyltrimethylammonium, and m-hydroxy phenylethyldimethylammonium. J. Pharmacol. 100, 382-392 (1950).

Marnay, A., and D. Nachmansohn: Sur la répartition de la cholinesterase dans le muscle couturier de la grenouille. C. R. Soc. Biol. (Paris) 125, 41-43 (1937).

Masland, R. L., and R. S. Wigton: Nerve activity accompanying fasciculation produced by prostigmine. J. Neurophysiol. 3, 269-275 (1940).

McCombie, H., and P. C. Saunders: Alkyl fluorophosphonates:

Preparation and physiological properties. *Nature (Lond.)* 157, 287-289 (1946).

Nachmansohn, D., and A. L. Machado: The formation of acetylcholine.

A new enzyme: "choline acetylase." *J. Neurophysiol.* 6, 397-403 (1943).

Nachmansohn, D., M. A. Rothenberg and E. A. Feld: Studies on cholin-

esterase. V. Kinetics of the enzyme inhibition. *J. biol. Chem.* 174,

247-256 (1948).

Nastuk, W. L.: The electrical activity of the muscle cell membrane at

the neuromuscular junction. *J. cell. comp. Physiol.* 42, 249-272 (1953).

Nastuk, W. L., and J. T. Alexander: The action of 3-hydroxyphenyl-

dimethylethylammonium (Tensilon) on neuromuscular transmission

in the frog. *J. Pharmacol.* 111, 302-328 (1954).

Oberst, F. W., and M. K. Christensen: Regeneration of erythrocyte

and brain cholinesterase activity in rats after sublethal exposures to

GB vapor. *J. Pharmacol. exp. Ther.* 116, 216-219 (1956).

Ogston, A. G.: Removal of acetylcholine from a limited volume by

diffusion. *J. Physiol.* 128, 222-224 (1955).

Randall, L. O.: Anticurare action of phenolic quaternary ammonium salts. J. Pharmacol. 100, 83-93 (1950).

Renshaw, R. R., P. F. Dreisbach, M. Ziff and D. Green: Onium compounds XIX. Thio-esters of choline and beta-methylcholine and their physiological activities. J. Am. Chem. Soc. 60, 1765-1770 (1938).

Richter, D., and P. G. Croft: Blood esterases. Biochem. J. 36, 746-757 (1942).

Riker, W. F.: Excitatory and anti-curare properties of acetylcholine and related quaternary ammonium compounds at the neuromuscular junction. Pharmacol. Rev. 5, 1-86 (1953).

Riker, W. F., and W. C. Wescoe: The direct action of prostigmine on skeletal muscle; its relationship to the choline esters. J. Pharmacol. 88, 58-66 (1946).

Riker, W. F., and W. C. Wescoe: The relationship between cholinesterase inhibition and function in a neuroeffector system. J. Pharmacol. exp. Ther. 95, 515-527 (1949).

Riker, W. F., J. Roberts, F. G. Standaert and H. Fujimori: The motor nerve terminal as the primary focus for drug induced facilitation of neuromuscular transmission. J. Pharmacol. 121, 286-312 (1957).

De Robertis, E., and H. S. Bennett: Some features of the submicroscopic morphology of synapses in frog and earthworm. J. biophys. biochem. Cytol. 1, 47-58 (1955).

De Robertis, E., A. Pellegrino de Iraldi, G. Rodriguez de Lores Arnaiz and L. Salganicoff: Cholinergic and non-cholinergic nerve endings in the rat brain. I. Isolation and subcellular distribution of acetylcholine and acetylcholinesterase. J. Neurochem. 9, 23-35 (1962).

De Robertis, E., G. Rodriguez de Lores Arnaiz, L. Salganicoff, A. Pellegrino de Iraldi and L. Zieher: Isolation of synaptic vesicles and structural organization of the acetylcholine system within brain nerve endings. J. Neurochem. 10, 225-235 (1963).

Sawyer C. H., C. Davenport and L. M. Alexander: Sites of cholinesterase activity in neuromuscular and ganglionic transmission. Anat. Rec. 106, 287-288 (1950).

Secher, O.: The peripheral action of ether estimated on isolated nerve muscle preparation III. Antagonistic and synergistic actions of ether and neostigmine. Acta pharmacol. Kbh. 7, 103-118 (1951).

Sherrington, C. S.: Remarks on some aspects of reflex inhibition. Proc. roy. Soc. B. 97, 519-545 (1925).

Smith, C. M., H. L. Cohen, E. W. Pelikan and K. R. Unna: Mode of action of antagonists to curare. J. Pharmacol. 105, 391-399 (1952).

Smith, C. M., J. C. Mead and K. R. Unna: Antagonism of tubocurarine. III. Time course of action of pyridostigmin, neostigmine and edrophonium in vivo and in vitro. J. Pharmacol. exp. Ther. 120, 215-228 (1957).

Straughan, D. W.: The release of acetylcholine from mammalian motor nerve endings. Brit. J. Pharmacol. 15, 417-424 (1960).

Tammelin, L. E.: Organophosphorylcholines and cholinesterases. Ark. Kemi 12, 287-298 (1958).

Volle, R. L., and G. B. Koelle: The physiological role of acetylcholinesterase (AChE) in sympathetic ganglia. J. Pharmacol. exp. Ther. 133, 223-240 (1961).

Wagner, R.: Neue Untersuchungen über den Bau und die Endigungen der nerven. Cited by Couteaux, R. (1955).

Waser, P. G.: XXI Int. Cong. of Physiol. Sciences. 30, (1959).

Werner, G.: Neuromuscular facilitation and antidromic discharges in motor nerves: their relation to activity in motor nerve terminals. J. Neurophysiol. 23, 171-187 (1960).

Wescoe, W. C., and W. F. Riker: The pharmacology of anticurare agents. Ann. N. Y. Acad. Sci. 54, 438-459 (1951).

Wilson, I. B.: The interaction of Tensilon and Neostigmine with acetylcholinesterase. Arch. int. Pharmacodyn. 104, 204-213 (1955).

Wilson, I. B., and F. Bergmann: Studies on cholinesterase. VII. The active surface of acetylcholine esterase derived from effects of pH on inhibitors. J. biol. Chem. 185, 479-489 (1950).

Wright, P. G.: An analysis of the central and peripheral components of respiratory failure produced by anticholinesterase poisoning in the rabbit. J. Physiol. (Lond.) 126, 52-70 (1954).

Zacks, S. I.: The Motor Endplate. W. B. Saunders Co. Phil. 1964

Zaimis, E. J.: The action of decamethonium on normal and denervated mammalian muscle. J. Physiol. 112, 176-190 (1951).


APPROVAL SHEET

The thesis submitted by John Goode has been read and approved by three members of the faculty of Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

16 January 1967
Date


Signature of Adviser